BEST AVAILA

Department Virol.

Subject Togavirus charactorizat

Name Hodgson

Address MIIB

Lab Notebook # 3425

National® Brand

43-648

Issued 5-4-92

Computation Notebook

Dennison Stationery Products Co., Framingham, MA 01701

75 Sheets
11¾"x 9¼"
4x4 Quad.

0 73333 43648 8

EXHIBIT T

BEST AVAILABLE COPY

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==03-MAY-1992=VEE26S=====================================PC/GENE===

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 361 AAACCAGGCA AGAGACAGCG CATGGTCATG AAATTGGAAT CTGACAAGAC GTTCCCAATC
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-ATCCGCCGCCACCATGGCT  856 → E3  III-VE3-F1 , III-VE3-F2
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 901 TGTGCTCAAC CACCAATTTG CTACGACAGA AAACCAGCAG AGACTTTGGC CATGCTCAGC
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1981 AGGAATTTTA CCGTCACCGA AAAAGGGTGG GAGTTTGTAT GGGGAAACCA CCCGCCGAAA
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2161 ATTGCAACCG TTTCCGTTGC AGCGTCTACC TGGCTGTTTT GCAGATCTAG AGTTGCGTGC
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           BamHI                    2302
-TCTAGGATCCGCCGCCACCATG  VE  6K   III-V6K-F1          2320
2281 TGCGCCCGCA CTGCCCGGGC CGAGACCACC TGGGAGTCCT TGGATCACCT ATGGAACAAT
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2401 CGCCTGCTCA GGTGCGTGTG CTGTGTCGTG CCTTTTTTAG TCATGGCCGG CGCCGCAGCG
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2461 CCGGCCTACG AGCACGCGAC CACGATGCCG AGCCAAGCGG GAATCTCGTA TAACACTATA
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                                              3699                3720
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     CATTAATAAT ATTAACCGAA CCACGACCGA TGATAACACC GGTACATGCA CGACTGGTTG   G
     ANCHOR                                      ANCHOR                  G
                                                                         C
3781 CAGAAACATA ATTGAATACA GCAGCAATTG GCAAGCTGCT TACATAGAAC TCGCGGCGAT   T
     GTCTTTGTAT TAACTTATGT CGTCGTTAAC CGTTCGACGA ATGTATCTTG AGCGCCGCTA   C
                                                                      clamp
3841 TGGCATGCCG CCTTAAAATT TTTATTTTAT TTTTCTTTTC TTTTCCGAAT CGGATTTTGT
     ACCGTACGGC GGAATTTTAA AAATAAAATA AAAAGAAAAG AAAAGGCTTA GCCTAAAACA
                                                            3891   III-VA-RI
3901 TTTTAATATT C
     AAAATTATAA G
           3911  TTCTAG ATCGGCTC
                  BglII   clamp

tal number of bases is: 3911.
A sequence composition:    1071 A;   1014 C;   982 G;   844 T;


quence name: 26X

=08-MAY-1992========================================PC/GENE===
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    GTCGGTTACG GGATAGCGTT GGGCAAGCGC CGGGGCGCGT CCGGGACCAA GGGGTCTTGG

121 GACCCTTTTC TGGCGATGCA GGTGCAGGAA TTAACCCGCT CGATGGCTAA CCTGACGTTC
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721 CTGGATAACC AGGGACGGGT GGTCGCTATT GTGCTGGGAG GTGTGAATGA AGGATCTAGG
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781 ACAGCCCTTT CAGTCGTCAT GTGGAACGAG AAGGGAGTTA CCGTGAAGTA TACTCCGGAG
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841 AACTGCGAGC AATGGTCACT AGTGACCACC ATGTGTCTGC TCGCCAATGT GACGTTCCCA
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901 TGTGCTCAAC CACCAATTTG CTACGACAGA AAACCAGCAG AGACTTTGGC CATGCTCAGC
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961 GTTAACGTTG ACAACCCGGG CTACGATGAG CTGCTGGAAG CAGCTGTTAA GTGCCCCGGA
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1441 GAACTCTATA CTCATCCCCC AGAACACGGA GTAGAGCAAG CGTGCCAAGT CTACGCACAT
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      2282        VE3-R2    2301 2302
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     CTTGTGAGAT AACACTGATG GCACATACAC TTACCTCTTT GAGGACACTT AAAGTTACCC

2941 GTCAAATTAA CTGCAGGTCC GCTTTCCACA GCTTGGACAC CCTTTGATCG CAAAATCGTG
     CAGTTTAATT GACGTCCAGG CGAAAGGTGT CGAACCTGTG GGAAACTAGC GTTTTAGCAC

3001 CAGTATGCCG GGGAGATCTA TAATTATGAT TTTCCTGAGT ATGGGGCAGG ACAACCAGGA
     GTCATACGGC CCCTCTAGAT ATTAATACTA AAAGGACTCA TACCCCGTCC TGTTGGTCCT

3061 GCATTTGGAG ATATACAATC CAGAACAGTC TCAAGCTCAG ATCTGTATGC CAATACCAAC
     CGTAAACCTC TATATGTTAG GTCTTGTCAG AGTTCGAGTC TAGACATACG GTTATGGTTG

3121 CTAGTGCTGC AGAGACCCAA AGCAGGAGCG ATCCACGTGC CATACACTCA GGCACCTTCG
     GATCACGACG TCTCTGGGTT TCGTCCTCGC TAGGTGCACG GTATGTGAGT CCGTGGAAGC

3181 GGTTTTGAGC AATGGAAGAA AGATAAAGCT CCATCATTGA AATTTACCGC CCCTTTCGGA
```

```
     CCAAAACTCG TTACCTTCTT TCTATTTCGA GGTAGTAACT TTAAATGGCG GGGAAAGCCT

3241 TGCGAAATAT ATACAAACCC CATTCGCGCC GAAAACTGTG CTGTAGGGTC AATTCCATTA
     ACGCTTTATA TATGTTTGGG GTAAGCGCGG CTTTTGACAC GACATCCCAG TTAAGGTAAT

3301 GCCTTTGACA TTCCCGACGC CTTGTTCACC AGGGTGTCAG AAACACCGAC ACTTTCAGCG
     CGGAAACTGT AAGGGCTGCG GAACAAGTGG TCCCACAGTC TTTGTGGCTG TGAAAGTCGC

3361 GCCGAATGCA CTCTTAACGA GTGCGTGTAT TCTTCCGACT TTGGTGGGAT CGCCACGGTC
     CGGCTTACGT GAGAATTGCT CACGCACATA AGAAGGCTGA AACCACCCTA GCGGTGCCAG

3421 AAGTACTCGG CCAGCAAGTC AGGCAAGTGC GCAGTCCATG TGCCATCAGG GACTGCTACC
     TTCATGAGCC GGTCGTTCAG TCCGTTCACG CGTCAGGTAC ACGGTAGTCC CTGACGATGG

3481 CTAAAAGAAG CAGCAGTCGA GCTAACCGAG CAAGGGTCGG CGACTATCCA TTTCTCGACC
     GATTTTCTTC GTCGTCAGCT CGATTGGCTC GTTCCCAGCC GCTGATAGGT AAAGAGCTGG

3541 GCAAATATCC ACCCGGAGTT CAGGCTCCAA ATATGCACAT CATATGTTAC GTGCAAAGGT
     CGTTTATAGG TGGGCCTCAA GTCCGAGGTT TATACGTGTA GTATACAATG CACGTTTCCA

3601 GATTGTCACC CCCCGAAAGA CCATATTGTG ACACACCCTC AGTATCACGC CCAAACATTT
     CTAACAGTGG GGGGCTTTCT GGTATAACAC TGTGTGGGAG TCATAGTGCG GGTTTGTAAA
                           K → ANCHOR
3661 ACAGCCGCGG TGTCAAAAAC CGCGTGGACG TGGTTAACAT CCCTGCTGGG AGGATCAGCC
     TGTCGGCGCC ACAGTTTTTG GCGCACCTGC ACCAATTGTA GGGACGACCC TCCTAGTCGG TAAT
 3661 VNOA-R1 3678 ACTTAAGCTTTCGGCTC  VNC
                  STOP   HIND III  clamp
3721 GTAATTATTA TAATTGGCTT GGTGCTGGCT ACTATTGTGG CCATGTACGT GCTGACCAAC
     CATTAATAAT ATTAACCGAA CCACGACCGA TGATAACACC GGTACATGCA CGACTGGTTG
               EI  VGA 3195
3781 CAGAAACATA ATTGAATACA GCAGCAATTG GCAAGCTGCT TACATAGAAC TCGCGGCGAT
     GTCTTTGTAT TAACTTATGT CGTCGTTAAC CGTTCGACGA ATGTATCTTG AGCGCCGCTA
               STOP
3841 TGGCATGCCG CCTTAAAATT TTTATTTTAT TTTTCTTTTC TTTTCCGAAT CGGATTTTGT
     ACCGTACGGC GGAATTTTAA AAATAAAATA AAAAGAAAAG AAAAGGCTTA GCCTAAAACA
                                                         3891
3901 TTTTAATATT C TTCGAA
     AAAATTATAA G AAGCTT T CGG CTC
      VA-R2 3911   Hind III  clamp

al number of bases is: 3911.
 sequence composition:    1071 A;   1014 C;   982 G;   844 T;

uence name: 26X

=21-SEP-1992==========================================PC/GENE===
```

Expression of VEE glycoprotein . . .

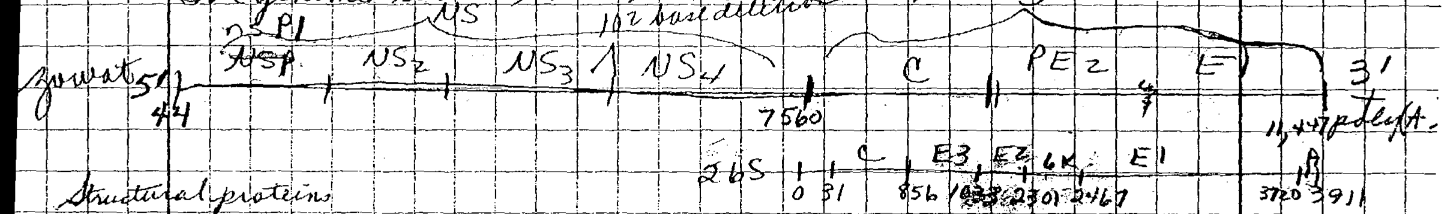
expression system.

Trinidad Donkey - TC

Do homology searches find ours

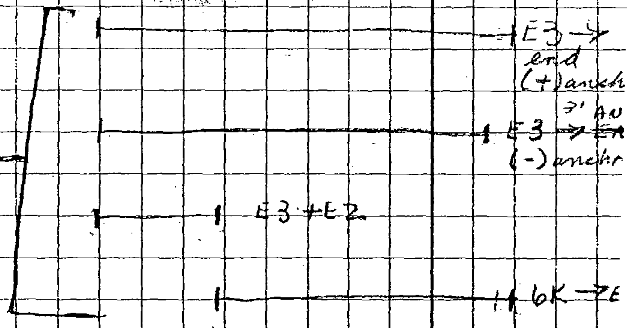
The entire sequence of VEE Strain TrD has been sequenced by Trent (Ref: Trent, D.W. 1991 Development of a genetically engineered Venezuelan equine encephalitis virus vaccine. Annual Report) Clones have been made by Nancy Davis (Ref: Virology 171, 189-204 (1989) In vitro Synthesis of Infectious Venezuelan Equine Encephalitis Virus RNA from a cDNA Clone: Analysis of a Viable deletion Mutant. Which are TrD. Zowat clones ~11,000 nucleotides - no sequence data. We have the sequence of VEE TrD of the 26S and non-structural regions. The 26S is 3911 bases in length. DNA sequence composition is 1071 A; 1014 C; 982 G; 844 T. 26S ~~message~~ message same as has (+) strand. There is a ORF starting at N.A. # 31 $\boxed{31}$ (ATG) and ends at 3793 (TGA) $\boxed{3795}$

The genome looks like this: ~~total~~ = 11,447



Structural proteins
C ~30
PE2 ~60
E1 ~55

Construct these glycoprotein segments



Will clone into pBlue Bac III baculovirus transfer vector. (Invitrogen Cat#

MCS =	React buffer	activity in hrs	Cleavage eff./ BPS from termini	Cleavage when 5' C residue is methyl cytosine	# of sites in TrD
Bam HI	3	1	+/- ①	+	0
Bgl II	3	1		+	0
Pst I	2	1	+ ①	–	3
Nco I	3	3		?	UC
Hind III	2	2	+/- ①	–	0

Will clone into the Bam HI and Bgl II sites.

Primers for cloning VEE virus glycoprotein structural genes into the Bam HI and Bgl II restriction endonuclease recognition sites of the baculovirus, pBlueBac III, transfer vector.

* VEE 26S: E3 - end (+ anchor):

17 OK III-VE3-F1: ✓

Bam HI "A" 5 → E3

5′ vcRNA: GGT CTA GGA TCC GCC GCC ACC ATG GCT TCA CTA GTG ACC ACC AT

clamp Kozak's seq 856 872

18 OK III-VA-R1: 34

Bgl II → END 26S

5′ vRNA: CTC GGC TAG ATC TGA ATA TTA AAA ACA AAA TCC G

clamp 3911 3891

* VEE 26S: E3 - end (- anchor):

III-VE3-F1

19 Redo III-VE2-R1: 35

Bgl II → BEFORE ANCHOR at END 26S

5′ vRNA: CTC GGC TAG ATC TGG CTG ATC CTC CCA GCA GGG AT

clamp 3620 3699

*VEE 26S: E3 + E2:

III-VE3-F1

20 Redo III-VE3-R1: 34

Bgl II → END of E2

5′-vRNA: CTC GGC TAG ATC TGG CCC GGG CAG TGC GGG CGC A

clamp 2301 2281

*VEE 26S: 6K to end (+ anchor):

III-V6K-F1: 43

21

Bam HI E → 6K

5′ vcRNA: GGT CTA GGA TCC GCC GCC ACC ATG GAG ACC ACC TGG GAG TCC T

clamp Kozak's seq 2302 2320

III-VA-R1

Expected PCR products (Kb)

VEE GP	PRIMERS	N.A. Location	Length of PCR product	
E3 → END (+) anchor	VE3-F1	27-856 = 829	3,095	
	VA-R1	3911 + 13 = 3,924		
E3 → END (-) anchor	VE3-F1	829	2,904	Repeat 5 June 92
	VE2-R1	3,720 + 13 = 3,733		
E3 + E2	VE3-F1	829	1,485	Repeat 5 June 92
	VE3-R1	2,301 + 13 = 2,314		
6K → END of E1	V6K-F1	2,302 - 24 = 2,278	1,646	
	VA-R1	3,911 + 13 = 3,924		

Have 3 minipreps of Zowat (5, 11, 12).

DUAL λ 02 Abs Factor= 1.000

19 May 92 5 ul in 500 ul H2O Read average = 01

S#	260.0	280.0	λA/λB	S#	260.0	280.0	λA/λB
Minipreps							
1	-0.0003	-0.0000	8.127				
2	0.7533	0.3714	2.028 Zowat 5				
3	0.5927	0.3116	1.902 " 11.				
4	0.5617	0.2949	1.905 " 12.				

λ	ABS	SOURCE	14:44	CELL
260.0	-0.0002	UV	05/19/92	1

DUAL λ 02 Abs Factor= 1.000

Read average = 01

S#	260.0	280.0	λA/λB	S#	260.0	280.0	λ
2 ul in 500 ul H2O = 1:251 dil							
1	~~0.0430~~	~~0.0390~~	~~1.102~~				
2	~~0.5154~~	~~0.3399~~	~~1.516~~				
3	~~0.6041~~	~~0.4424~~	~~1.365~~				
4	~~0.4269~~	~~0.3309~~	~~1.290~~				
5	~~0.2151~~	~~0.1530~~	~~1.405~~				
6	0.0000	0.0001	.2578				
7	0.4743	0.3020	1.570 VAR1 21MY92				
8	0.5629	0.4048	1.390 VE3R1 " ← REDON				
9	0.3850	0.2928	1.314 VE2R1 " ← REDON				
10	0.1734	0.1149	1.510 VKRF1 "				

4JUN92

100 ul total volume

0,2404 0.1441 1.668 VE3F1 - from notebook # 3426 p15

λ	ABS	SOURCE	10:25	CELL
260.0	0.0001	UV	05/22/92	1

```
                    - 394 Synthesis Setup Listing -          (Version 1.01)

Column 3                                               10:50:58A , 5/13/92

Run ID   :
Cycle    : Cyc02 user
End Proc: End CESS       (DMT = On )
Sequence: VE3F1

Total bases =  44
          A=  9, G= 10, C= 16, T=  9, 5=  0, 6=  0, 7=  0, 8=  0
          (mixed bases=  0)

MW:   14348.8

     5'> GGT CTA GGA TCC GCC GCC ACC ATG GCT TCA CTA GTG ACC

        ACC AT  <3'
```

$E_m = A(16000) + C(7000) + G(12000) + T(9600)$

$= 9(16000) + 16(7000) + 10(12000) + 9(9600)$

$= 144{,}000 + 112{,}000 + 120{,}000 + 86{,}400$

$E_m = 462{,}400$

$$\text{Molarity} = \frac{OD_{260} \cdot \text{Dil factor}}{E_m}$$

$$= \frac{0.2404 \cdot 101}{462{,}400}$$

$$= 0.00005251$$

Molarity = 52.51 pmol/ul

PCR; Need 100 pmol/ul

Use 1.9 ul [illegible]

PCR – 22 May 92

DNA →	λC	ZOWAT #11			
PCR PRIMERS (100 pmol/ul)	Pr#1 Pr#2	VE3-F1 VA-R1	VE3-F1 VE2-R1	VE3-F1 VE3-R1	V6K-F1 VA-R1
Expected length →		#1 3,095	#2 2,904	#3 1,485	#4 1,646
H_2O (ul)	62	70.3	70.2	70.4	71.1
Template (ul) (Minipreps)	1	0.5	0.5	0.5	0.5
Pr#1 (ul) or FORWARD PR.	5	1.9	1.9	1.9	1.05
Pr#2 (ul) OR REVERSE PR.	5	0.31	0.38	0.25	0.31
10X RXN Buffer 10mM dNTP mix	M.M.	26 ul of M.M.			
Taq pol (ul)	1	1 29.71 100.01	1 29.78 99.98	1 29.65 100.05	1 28.86 99.9
	Vortex, microfuge				
Mineral oil (ul)	100	→			

Thermocycler Program – User 04 file 40

Step-cycle: 30 sec @ 95°C
30 sec @ 52°C
3 min @ 72°C } 35 cycles

Delay cycle: 10 min @ 72°C

Soak cycle: 4°C

dNTP mix – PE Cetus dNTPs

25 ul dATP
" dCTP
" dGTP
" dTTP
100 ul H_2O
200 ul TV

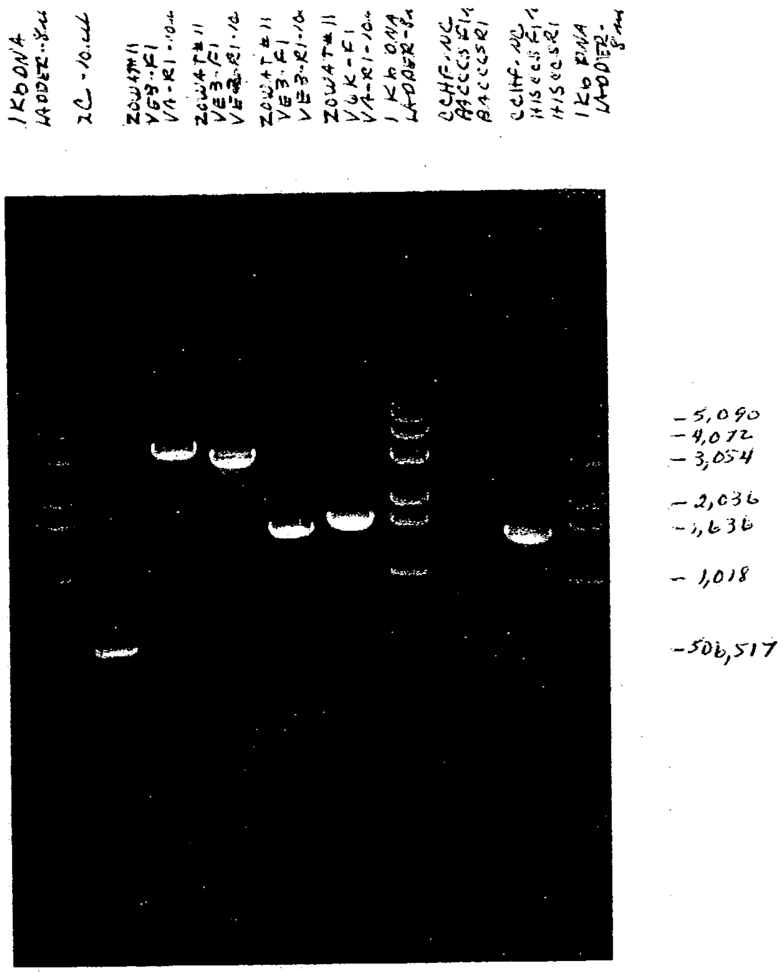
Master mix

10X Rxn Buffer = 10 X 6 = 60 ul

10mM dNTP mix = 16 X 6 = 96 ul

156 ul

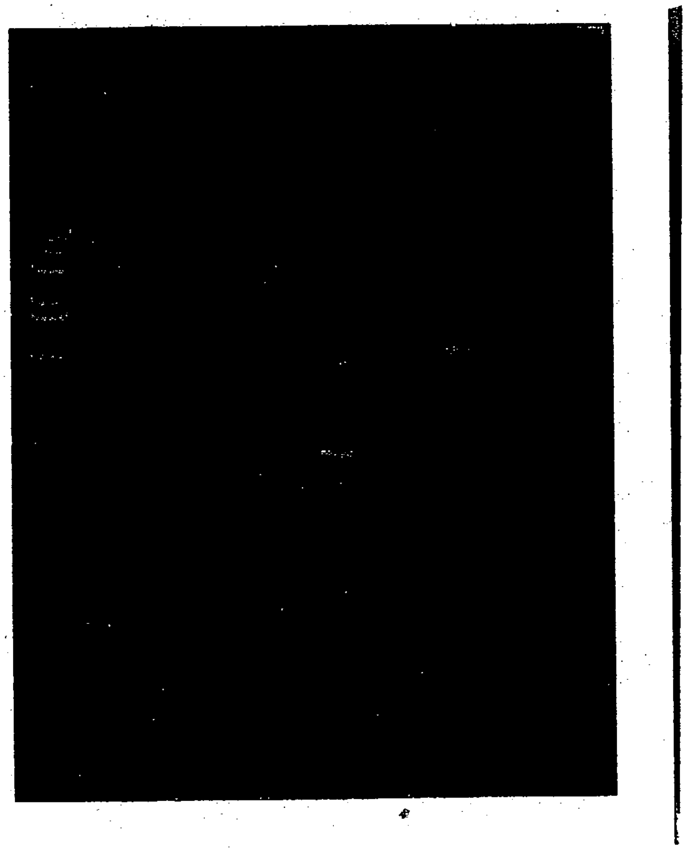
22MAY92



1Kb DNA LADDER-8ul
2C-10ul
ZOWAT#11 VG9-RI VA-RI-10ul
ZOWAT#11 VE3-RI VE3-RI-10
ZOWAT#11 VE3-RI VE3-RI-10
ZOWAT#11 VK-RI VA-RI-10ul
1Kb DNA LADDER-8ul
CCHF-NC BACCCS-RI BACCCS-RI
CCHF-NC HISCCS-RI HISCCS-RI
1Kb DNA LADDER-8ul
-5,090
-4,072
-3,054
-2,036
-1,636
-1,018
-506,517

1% LE agarose
100 V

26MAY92 1. Excised bands from gel



26MAY92 2. Weight of band = 0.35 g

1g = 1 ml

Used 1.5 ml tubes

3. Add 2.5 - 3 volumes of NaI = 1.1 ml of NaI
4. Incubate 55°C for ~5 min
5. Added 12 ul of Glass Milk. Place on ice for 5 min.
6. Centrifuge 5 sec. Discard NaI sol supernatant.
7. Wash pellet 4X w/ 400 ul New Wash each wash. Pellet at 5 sec.
8. Elute DNA w/ 2X 5 ul H_2O.

5X amp 26MAY92

DNA →	λC	20WAT #11 / Gel banded / GC of 26MAY92			
PCR PRIMERS	Pr#1 Pr#2	VE3-F1 VA-R1	VE3-F1 VE2-R1	VE3-F1 VE3-R1	V6K-F1 VA-R1
		#1	#2	#3	#4
H_2O (ul)	62	351.5	351	352	355.5
Template (ul) Gel banded, GC	1	2.5	2.5	2.5	2.5
Pr#1 (ul) or FORWARD PR.	5	9.5	9.5	9.5	5.25
Pr#2 (ul) or REVERSE PR.	5	1.55	1.9	1.25	1.55
10X Rxn buffer 10 mM dNTP mix	26	130	130	130	130
Taqpol (ul)	1	5	5	5	5
		500.0			

Vortex, microfuge

Mineral oil (100ul) 100ul

dNTP mix - PE Cetus dNTP's

62.5 ul dATP

" ul dCTP

" ul dGTP

" ul dTTP

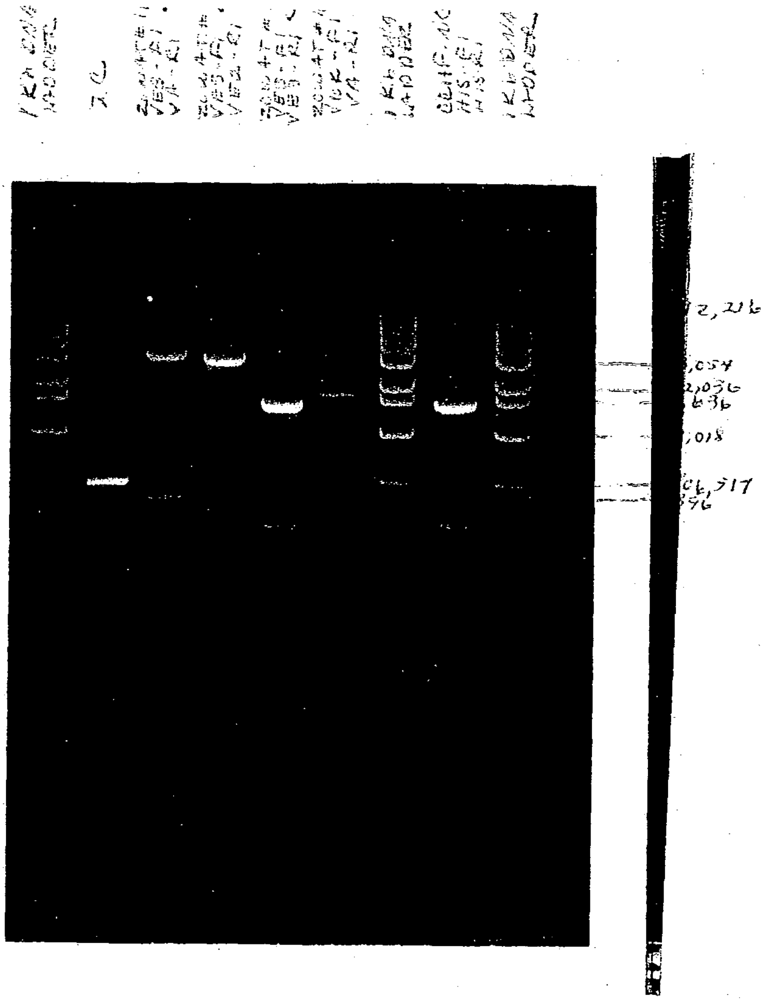
250 ul H_2O

Master Mix

10X Rxn buffer = 27 x 10 = 270 ul

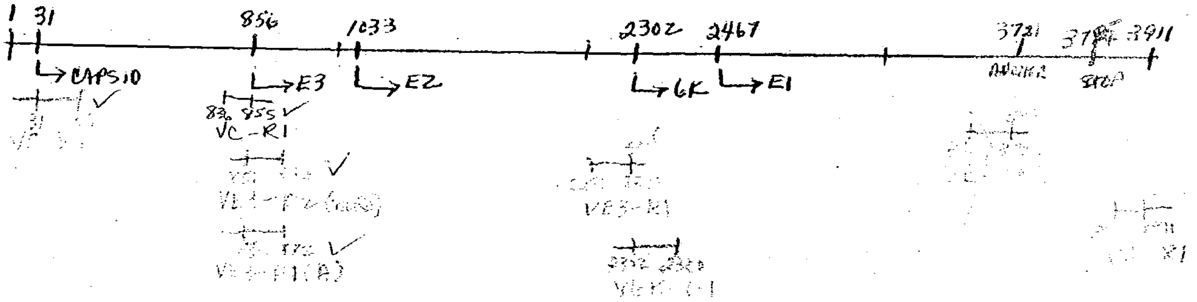
10 mM dNTP mix = 27 x 16 = 432 ul

27MAY92



1.2% AGAROSE GEL

26S



VC-F1
5'>GGT CTA GGA TCC GCC GCC ACC ATG TTC CCG TTC CAG CCA ATG <3'
clamp BamHI Kozak's seq CAPSID

VE3-F2
856
5'>GGT CTA GGA TCC GCC GCC ACC ATG TCA CTA GTA CCA CCA T <3'
clamp BamHI Kozak's seq E3

VE2-R1
Bgl II A
5'>CTC GGC TAG ATC TAA TGG GTG ATC CTC CCA GCA GGG AT<3'
clamp STOP 3720 E1 3699

VE3-R1
Bgl II
5'>CTC GGC TAG ATC TAA TGG CCC GGG CAG TGC GGG CGC A<3'
clamp STOP 2301 E2 2281

```
        DUAL λ        02          Abs       Factor= 1.000
                                            Read average = 01
 S#      260.0     280.0     λA/λB    S#     260.0     280.0    λA/λB
         5ul in 500ul H2O = 1:101 dil      200ul total volume
 1       0.0009    0.0007    1.200
 2       0.5566    0.3374    1.649 VE2-R1  4Jun92
 3       0.8069    0.5146    1.568 VE3-R1   "
 4       0.3637    0.2892    1.257 VC-F1    "
 5       0.2651    0.1893    1.400 VE3-F2   "

    λ        ABS      SOURCE      16:21     CELL
 260.0     0.0015      UV      06/08/92      1
```

```
                       - 394 Synthesis Setup Listing -        (Version 1.01)

Column 1                                                 11:06:40A , 6/ 4/92

Run ID  :
Cycle    : Cyc02 user
End Proc: End CESS       (DMT = On )
Sequence: VE2R1

Total bases =  38
        A=  8, G= 10, C= 11, T=  9, 5=  0, 6=  0, 7=  0, 8=  0
        (mixed bases=  0)

MW:  12471.6

    5'> CTC GGC TAG ATC TAA TGG CTG ATC CTC CCA GCA GGG AT  <3'
```

$E_m = A(16000) + C(7000) + G(12000) + T(9600)$

$= 8(16000) + 11(7000) + 10(12000) + 9(9600)$

$= 12{,}8000 + 77{,}000 + 120{,}000 + 86{,}400$

$E_m = 411{,}400$

$\text{Molarity} = \dfrac{OD_{260} \cdot \text{Dil factor}}{E_m}$

$= \dfrac{0.5566 \cdot 101}{411{,}400}$

$= 0.00013665$

Molarity = 136.65 pmol/ul

PCR: Need 100 pmol/ul

Use 0.73 ul / rxn

12/02/92

Sequen

Expected PCR product (Kb)

VEE PROTEIN(S)	PRIMERS	N.A. LOCATION	LENGTH of PCR PRODUCT	
ENTIRE 26S	VC-F1	31-21=10	3,914	✓
	VA-R1	3911+13=3,924		
E3, E2, 6K, E1, ANCHOR	(A) VE3-F1	856-27=829	3,095	
	VA-R1	3911+13=3924		
E3, E2, 6K, E1, ANCHOR	VE3-F2	856-24=832	3,092	
	VA-R1	3911+13=3924		
E3, E2, 6K, E1	VE3-F2	856-24=832	2,904	
	VE2-R1	~~24~~ 3720+16=3736		
E3, E2	VE3-F2	856-24=832	1,485	
	VE3-R1	2301+16=2317		

PCR AMPLIFICATION - 15JUN92

ZOWAT #11

DNA	λC	①	②	③	④	⑤
PCR PRIMERS	Pr#1	VE3-F2	VE3-F2	VC-F1	VE3-F1	VE3-F2
	Pr#2	VE2-R1	VE3-R1	VA-R1	VA-R1	VA-R1
Expected length		2,904	1,485	3,914	3,095	3,092
H_2O (ul)	62	70.2	70.4	71.0	70.3	70.6
Template (ul) Miniprep	1	0.5	0.5	0.5	0.5	0.5
Pr#1 (ul) or FORWARD PR.	5	1.58	1.58	1.17	1.9	1.58
Pr#2 (ul) or REVERSE PR.	5	0.73	0.49	0.3	0.3	0.3
10X RXN Buffer }	M.M	26 ul of M.M				
10mM dNTP mix }						
Taq pol (ul)	1	1	1	1	1	1
	100	29.81 100.01	29.57 99.97	28.97 99.97	29.7 100	29.38 99.98
Mineral oil (ul)	100 →					

Vortex, microfuge

Thermocycler Program

Step cycle: 30 sec @ 95°C
30 sec @ 57°C
3 min @ 72°C } 35 cycles

Delay cycle: 10 min @ 72°C
Soak cycle: 4°C

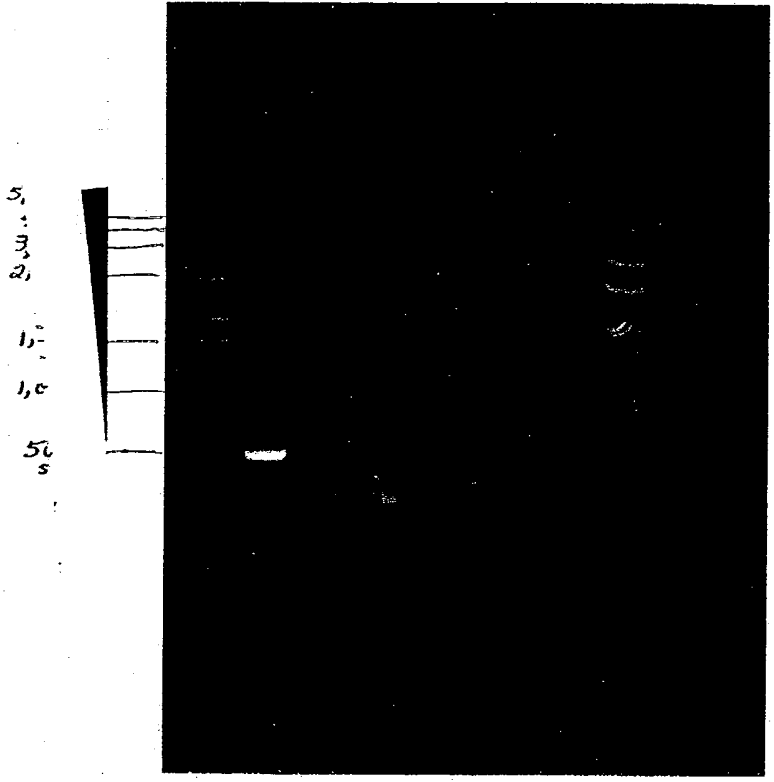
dNTP mix - PE Cetus dNTPS

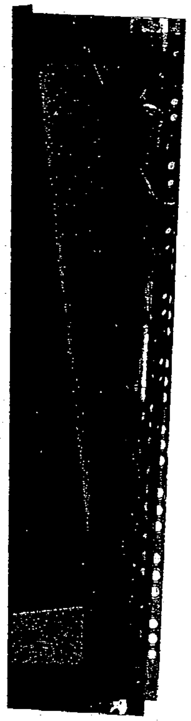
25 ul dATP
" dCTP
" dGTP
" dTTP
100 ul H_2O

Master mix

10X Rxn buffer = 10 × 7 = 70 ul
10 mM dNTP mix = 16 × 7 = 112 ul

16JUN92 0.8% LE agarose gel 70V





16JUN92 1. Excised bands from gel.
2. Weigh of band = 0.2 g
3. Add 2.5 - 3 X volumes of NaI = 600 ul
4. Incubate 55°C for 5 min.
5. Add 12 ul of Glass milk. Ice for 5 min.
6. Centrifuge 5 sec. Discard NaI sol supernatant
7. Wash pellet 4 X w/ 400 ul New Wash each wash
8. Pellet for 5 sec
9. Elute DNA w/ 2X 5 ul H_2O
10. Add 10 ul H_2O

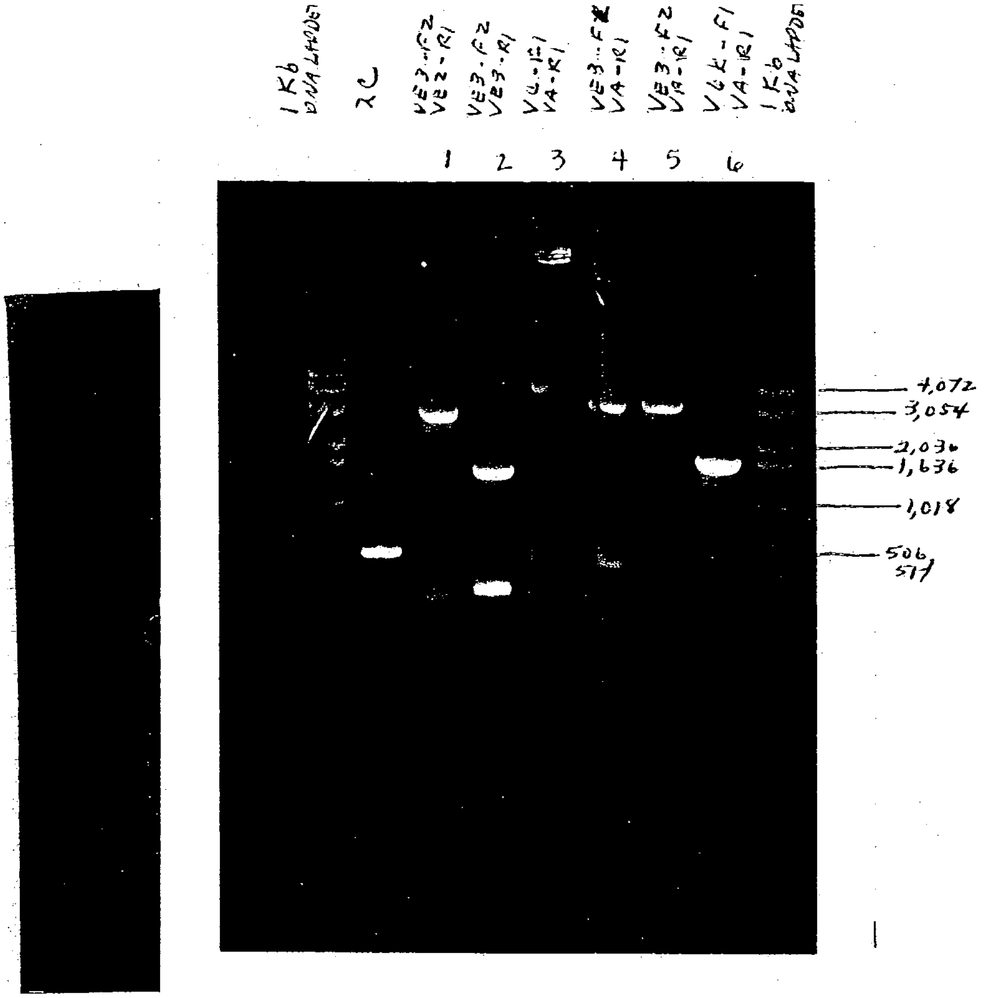
5x PCR amplification - 16JUN92

DNA →	λC	20 WAT #11 → PCR ampl. / G-B/GC of 16JUN92					
PCR PRIMERS	Pr#1 Pr#2	VE3-F2 VE2-R1	VE3-F2 VE3-R1	VC-F1 VA-R1	VE3-F1 VA-R1	VE3-F2 VA-R1	V6K-A VA-R1
		①	②	③ ✓	④ ✓	⑤ ✓	⑥
Expected lengths		2,904	1,485	3,914	3,095	3,095	1,646
H₂O (ul)	62	343.45	344.65	347.65	344	345.6	353.2
Template (ul)	1	10	10	10	10	10	5
Pr#1 (ul) or FORWARD PR.	5	7.9	7.9	5.85	9.5	7.9	5.25
Pr#2 (ul) or REVERSE PR.	5	3.65	2.45	1.5	1.5	1.5	1.55
10X Rxn Buffer	MM 26	130	130	130	130	130	130
10mM dNTP mix							
Taq pol (ul)	1	5 156.55 500	5 155.35 500	5 152.35 500	5 156 500	5 154.4 500	5 146.8 500
	Vortex, microfuge						
Mineral oil (ul)	100 →						

Thermocycler Program
Stepcycle: 30sec @ 95°C
30sec @ 52°C } 30 cy
3min @ 72°C
Delay cycle: 10 min @ 72°C
Soak cycle: 4°C

Master Mix
10X Rxn buffer = 35 x 10 = 350 ul
10mM dNTP mix = 35 x 16 = 560 ul

dNTP mix - PE Cetus dNTPS
70 ul d ATP
" d CTP
" d GTP
" d TTP
280 ul H₂O
560



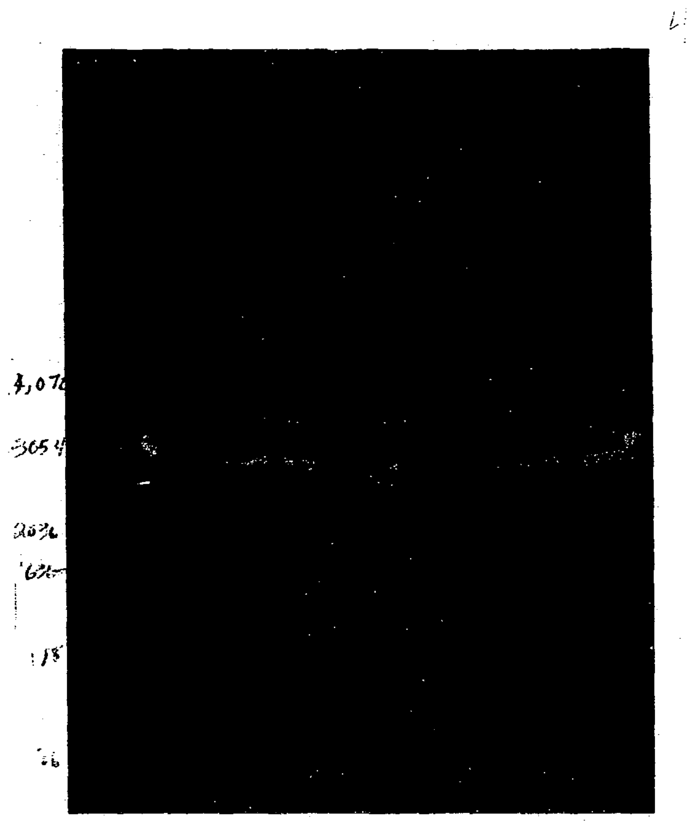
Expected lengths
1 - 2,904
2 - 1,485
3 - 3,914
4 - 3,095
5 - 3,095
6 - 1,646

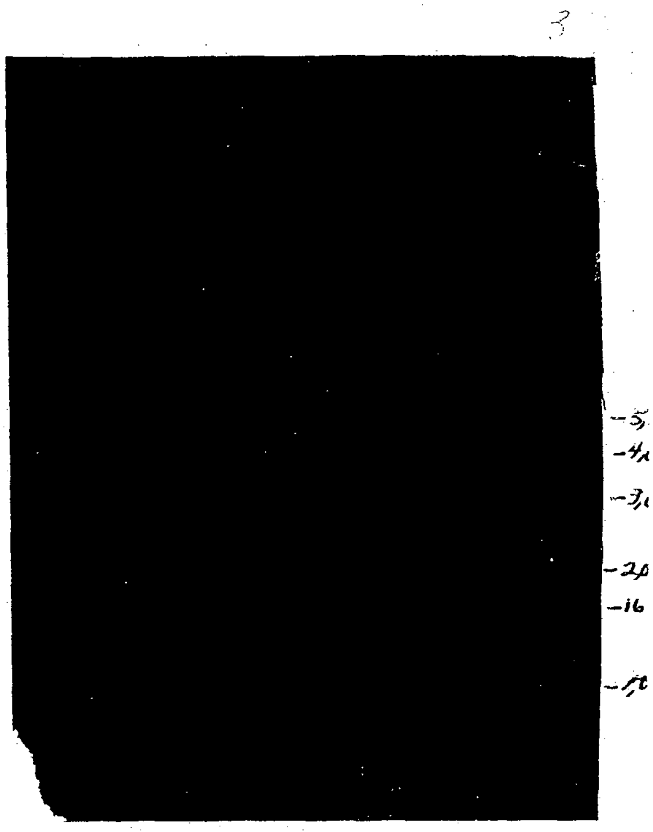
15 Jun 92
1. Excised bands from gel
2. Weigh of band = ~0.55g
3. Add 2.5-3x volumes of NaI = 1.4ml of NaI

0.8% LE agarose gels MPH w/

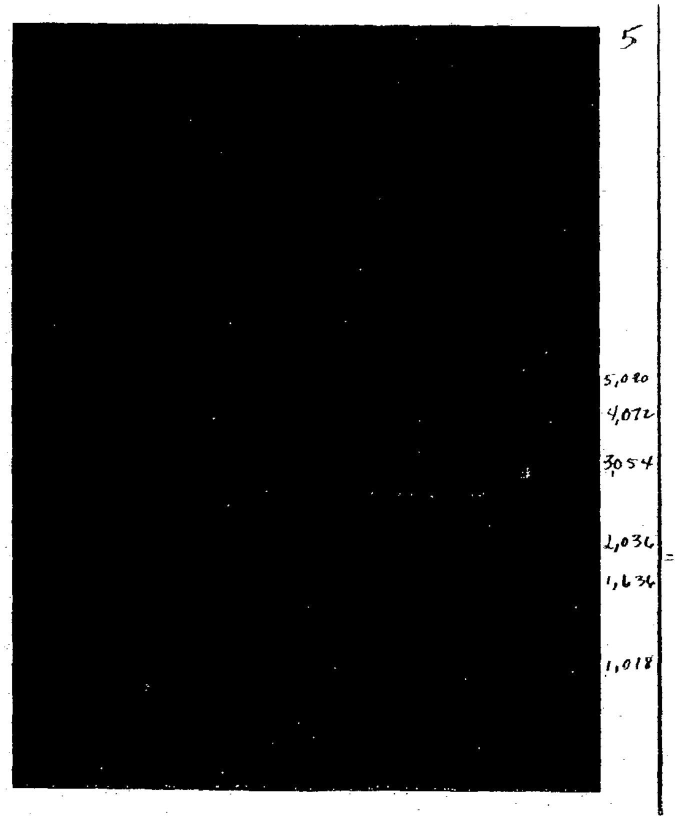
#4 ZWAT #11 : VE3-F1 3.095Kb
VA-R1

#3 ZCWAT #11 : VC-F1 3,914Kb
VA-R1





#5 ZCWAT #11 : VE3-F2 3.095Kb
VA-R1



```
        DUAL λ      02         Abs     Factor= 1.000
                                       Read average = 01
S#    260.0    280.0   λA/λB    S#    260.0    280.0   λA/λB
      Stock in 50ul H2O
3     0.0004   0.0003  1.494
4  3  0.0184   0.0113  1.630  92.9ug/ml =   0.093ug/ul
5  4  0.0193   0.0116  1.665  97.5ug/ml =   0.097ug/ul
6  5  0.0264   0.0128  2.069  133.3ug/ml =  0.13 ug/ul
================================================================
  λ       ABS      SOURCE    12:14   CELL
260.0    0.0005   UV/Vis   06/16/92   1
```

36

VC-R1

5>CTC GGC TAG ATC TAA TCC ATT GCT CGC AGT TCT CCG

clamp BglII STOP 855 CAPSID 834

```
      DUAL λ      02        Abs      Factor= 1.000
                                     Read average = 01
 S#    260.0    280.0    λA/λB    S#    260.0    280.0    λA/λB
```

5ul in 500ul H_2O = 1:101 dil

```
 1     0.0006   0.0001   4.429
 2     0.4969   0.2970   1.672
```

VC-R1

2509.345 ug/ml = 2.51 ug/ul

```
 λ       ABS      SOURCE     10:26     CELL
260.0    0.0005    UV       06/17/92    1
```

Expected PCR Product

VEE PROTEIN	PRIMERS	N.A. LOCATION	LENGTH of PCR PRODUCTS(
CAPSID	VC-F1	31-21=10	861 Kb
	VC-R1	855+16=871	

16

```
                 - 394 Synthesis Setup Listing -          (Version 1.01)

Column 1                                              11:25:12A , 6/15/92

Run ID   :
Cycle    : Cyc02 user
End Proc: End CESS      (DMT = On )
Sequence: VCR1

Total bases =  36
         A=  6, G=  7, C= 12, T= 11, 5=  0, 6=  0, 7=  0, 8=  0
         (mixed bases=  0)

MW:  11690.2

     5'> CTC GGC TAG ATC TAA TCC ATT GCT CGC AGT TCT CCG <3'
```

Em = A(16000) + C(7000) + G(12000) + T(9600)

= 6(16000) + 12(7000) + 7(12000) + 11(9600)

= 96,000 + 84,000 + 84,000 + 105,600

Em = 369,600

$$\text{Molarity} = \frac{OD_{260} \times \text{Dil factor}}{Em}$$

$$= \frac{0.4969 \times 101}{369,600}$$

= 0.00013601

Molarity = 136.01 pmol/ul

PCR: Need 100 pmol/ul

Use 0.74 ul/rxn.

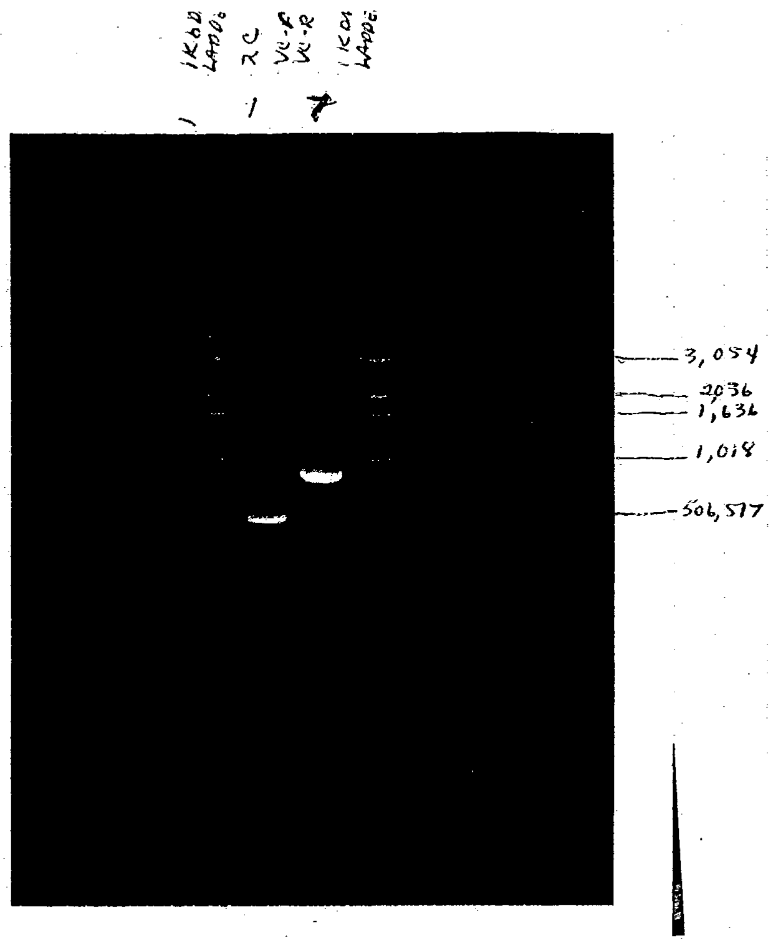
PCR-AMPLIFICATION - 17JUN92

DNA	λC	ZOWAT #11
PCR PRIMERS	Pr#1	VC-F1
	Pr#2	VCR1 #7
Expected length (kb)		861
H₂O (ul)	62	70.6
Template (ul) (miniprep)	1	0.5
Pr#1 (ul) or FORWARD PR.	5	1.17
~~Pr~~ Pr#2 (ul) or REVERSE PR.	5	0.74
10x RXN Buffer	26 MM	.26
10 mM dNTP mix		
Taq pol (ul)	1	1 29.41
	Vortex, microfuge	
Mineral oil (ul)	100 →	

Thermocycler Program
Step cycle: 30 sec @ 95°C
30 sec @ 52°C } 30 cycles
3 min @ 72°C
Delay cycle: 10 min @ 72°C
Soak cycle: 4°C

dNTP mix - PE Cetus dNTPs
12.5 ul dATP
" " dCTP
" " dGTP
" " TTP
50 " H₂O

Master Mix
10X Rxn Buffer = 10 x 3 = 30
10 mM dNTP mix = 16 x 3 = 48



Expected length (Kb)

1 -
7 - 8612

5X AMP - 17JUN92

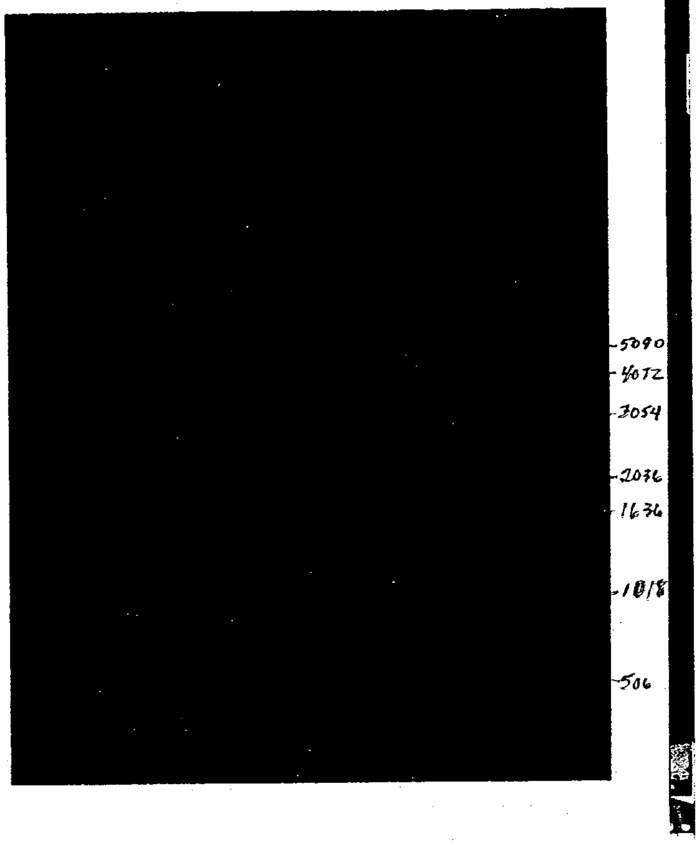
DNA	RC	ZOUAT #11
PCR PRIMERS	Pr#1	VC-F1
	Pr#2	VC-R1
Expected length (KB)		861
H_2O (ul)	62	353
Template (ul) (miniprep)	1	2.5
Pr#1 (ul) or FORWARD PR.	5	5.85
Pr#2 (ul) or REVERSE PR.	5	3.1
10x RXN Buffer	26	130
10 mM dNTP mix	MM	
Taq pol (ul)	1	5
Mineral oil	100ul →	50.05

Thermocycler Program
Step cycle: 30 sec @95°C
30 sec @52°C } 30c
2 min @ 72°C
Delay cycle: 10 min @ 72°C
Soak cycle: 4°C

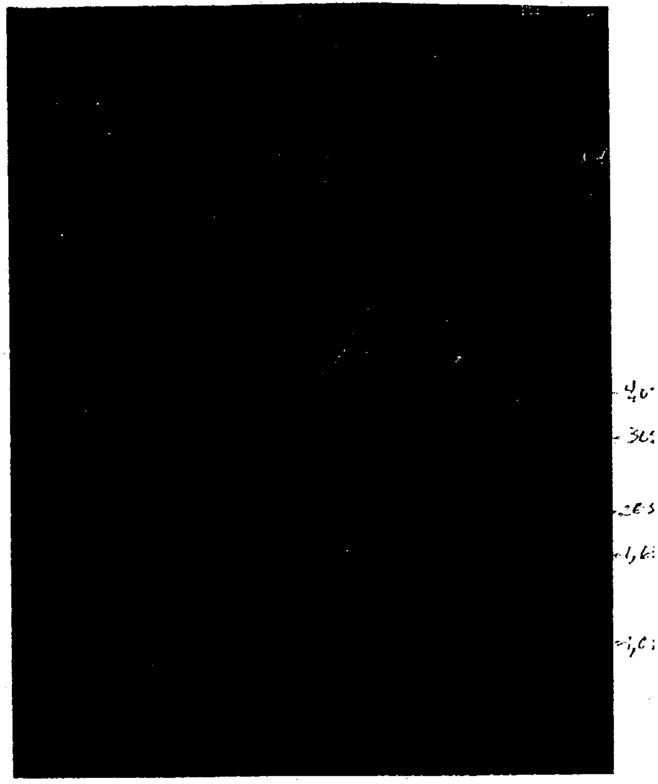
dNTP mix - PE Cetus dNTPs
25 ul dATP
" " dCTP
" " dGTP
" " dTTP
100 " H_2O

Master Mix
10X Rxn Buffer = 7x 10 = 70
10 mM dNTP mix = 7x 16 = 112

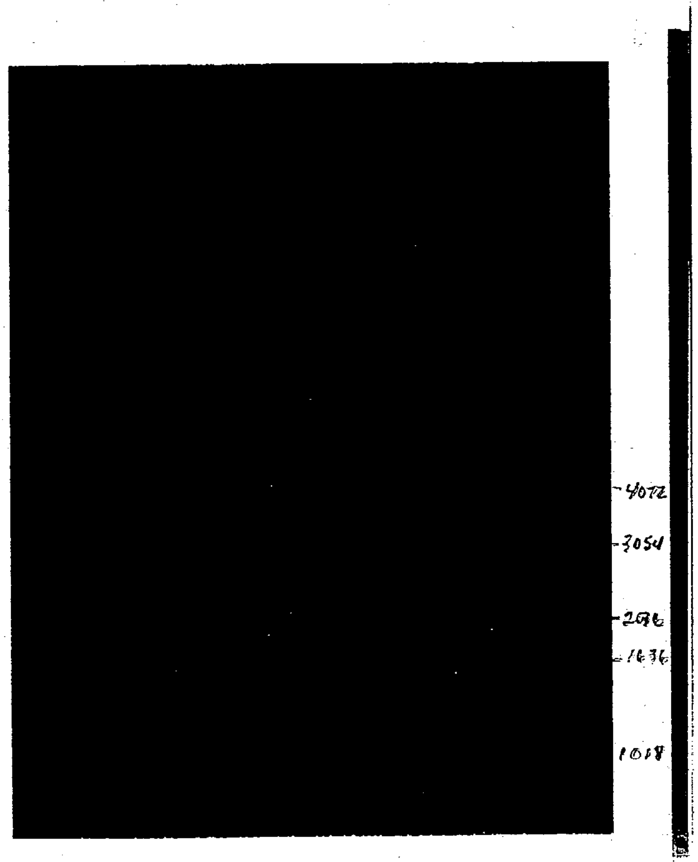
ZCWAT#11: VE3-F2 Expected length: 1,404
VE3-R1



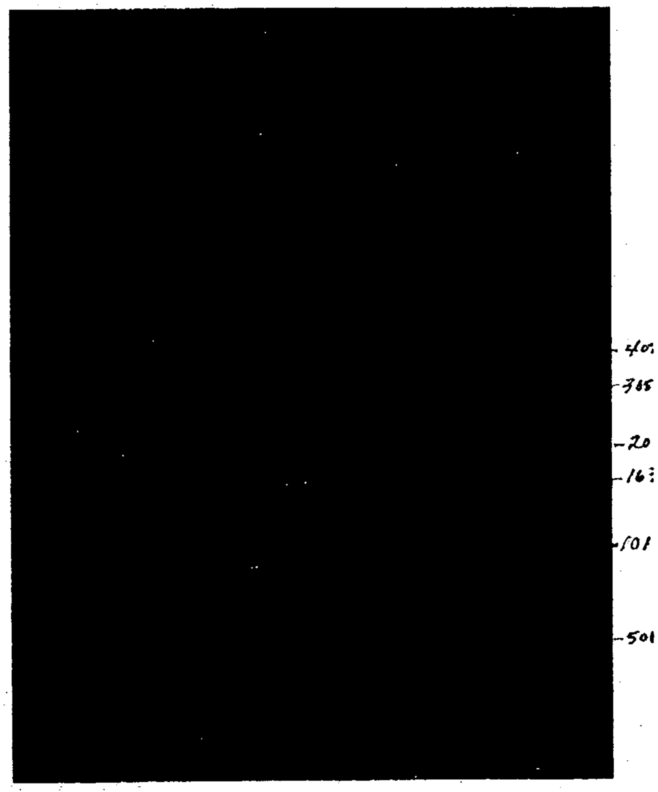
ZCWAT#11: VE3-F6 Expected length: 1,483
VE3-R1



ZCWAT#11: VGK-F1 Expected length: 1,646
VA-R1



ZCWAT#11: VC-F1 Expected length: 861
VC-R1

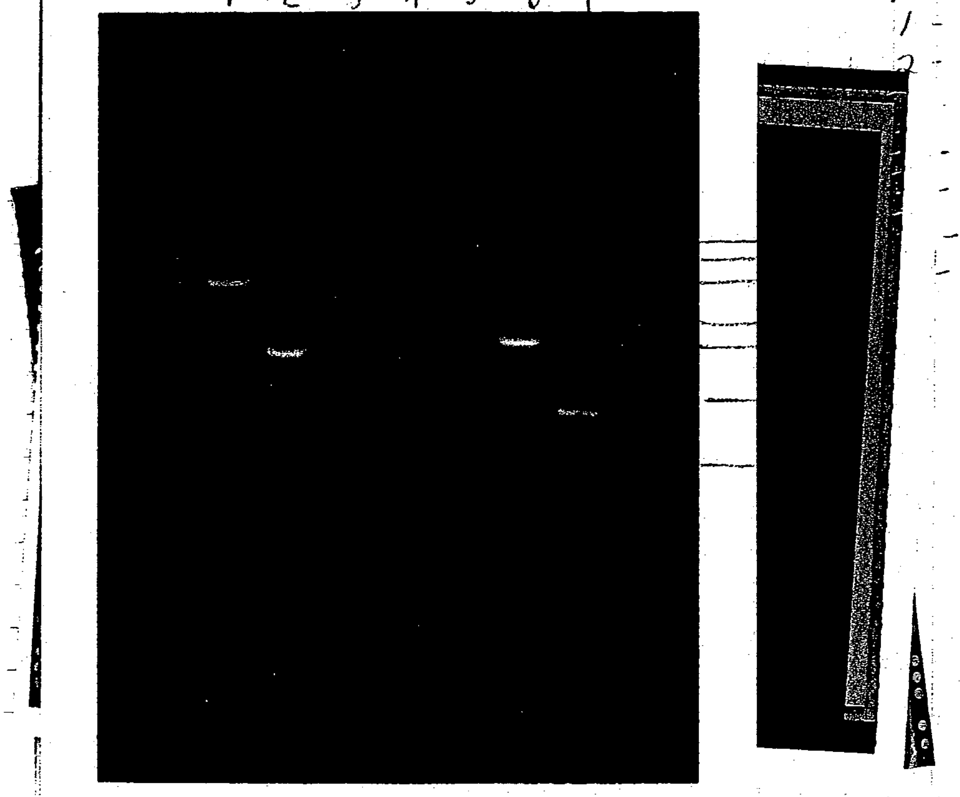


```
        DUAL λ        02           Abs       Factor= 1.000
                                             Read average = 01
  S#     260.0     280.0     λA/λB     S#     260.0      280.0     λA/λB
         50ul sample

  1      1.1650    0.5889    1.978  1
  2      1.2103    0.6227    1.943  2  60 ng/ml = 0.06 ug/ul
  3      1.0947    0.5626    1.945  6
  4      0.9040    0.4532    1.994  7. 45.2 ug/ml = 0.0452 ug/ul
  5      0.0099    0.0100    .9857  Blank
```

Can detect 10 ng w/ EtBr gel

```
=====================================================================
   λ          ABS        SOURCE       14:03      CELL
 260.0       0.0104        UV       06/19/92     sgle
```



Expected length (Kb):
1 - 2,904
2 - 1,485
3,914
- 3,095
- 3,095
- 1,646
- 861

DUAL λ 02 Abs Factor= 1.000

Read average = 01

S#	260.0	280.0	λA/λB	S#	260.0	280.0	λA/λB

55 ul sample – VEE - 26S + VEE-GP

1 ~~0.0486 0.0470 1.033~~ #3 = VEE26S

2 ~~0.2514 0.1480 1.698~~ #5 = VEEGP > Dig w/ BamHI + BglII, Cut100

3 ~~0.7103 0.4541 1.564~~

4 0.0090 0.0087 1.032 Blank (H2O)

5 0.2370 0.1335 1.775 #3 11,85 ug/ml = 0.01185 ug/ul = 0.5925 ug in sample

6 0.7023 0.4459 1.575 #5 35.115 ug/ml = 0.035115 ug/ul = 1.931325 ug in sample

~~3 digested w~~/

```
==============================================================
 λ        ABS     SOURCE    10:50   CELL
260.0    0.7037   UV/Vis  09/14/92  sgle
```

Digestion of VEE-26S, VEE-GP, ~~Sindbis-GP (SV-GP)~~ with Bam HI and Bgl II

1SEP92/ 10:30A CBCC 150µNR

1. Set up the following digestions:

#3 VEE-26S / 50 ul of PCR DNA (4.65ug VEE 26S, 6.5ug VEE-GP.
#5 VEE-GP 10 ul of React 3 buffer

VCF1 VAR1 #3 VEE-26S: 0.093ug/ul 2.5 ul Bam HI (10U/ul)
VCB F2 VAR1 #5 VEE-GP: 0.13ug/ul 2.5 ul Bgl II (10U/ul)
30 ul H_2O

~~Sindbis GP(SV-GP) 50ul of PCR DNA~~
~~10 ul of React 3 buffer~~
~~2.5 ul Bam HI (10U/ul)~~
~~2.5 ul Bgl II (10U/ul)~~
~~35 ul H_2O~~

2. Digest at 37°C for 4 hr. Heat 65°C for 20 min Quench on ice for 10 min.

3. Extract ~~twice~~ twice with an equal volume of Tris-HCl, 1mM, pH 8.0 saturated phenol / $CHCl_3$ / isoamyl alcohol (25:24:1). Vortex 1 min. Microfuge 12,000xg for 2 min. Back extract 1X. Extract twice with equal volume of $CHCl_3$.

Back extract w/50ul 50mM Tris-HCl pH 8.0

4. Precipitate with 1/10 volume of 3M NaOAc, pH 5.2 and 2 volumes of 100% EtOH.

5. Freeze on dry ice for 15 min or ON @ -20°C. Microfuge for 15 min @ 4°C.

6. Wash the pellet with 100ul 80% EtOH. Freeze on dry ice for 5 min. Microfuge for 5 min @ 4°C

7. Resuspend the DNA with ~~[illegible]~~ water to 500 ul. Heat for 10 min @ 45°C

8. Concentrate and remove the cut ends with a Centricon 100 @ 20°C. Insert sample reservoir into filtrate cup. Pre-rinse the membrane to get rid of the azide by centrifuging 2 ml of deionized H_2O through the device. Spin device in the concentration mode until at least half the rinse passes into the filtrate cup (~5 min). then invert device and centrifuge @ 2500 rpm for 2 min to remove remaining rinse. Add DNA solution to sample reservoir. Centrifuge the Centricon 100 concentrator at 2500 rpm for 45 min in a Sorvall SS-34 rotor. Dilute to 500 ul with ~~cold~~ deionized H_2O and centrifuge again for 15 min. Repeat last step. Add retentate cup / invert unit and centrifuge at 2500 rpm for 2 min to transfer concentrate into cup.

9. Transfer retentate to Eppendorf tubes. Quantitate @ $A_{260/280}$ (A_{260} = 50ug/ml). Speed vac entire sample to dryness.

GIBCO BRL
LIFE TECHNOLOGIES, INC.
GAITHERSBURG, MARYLAND 20898

5213SA
Lot No. CCA101
Bgl II

5'-A↓GATC T-3'
3'-T CTAG↑A-5'

400 units; 10 U/µl
Store at -20°C (not frost-free)

Description:
Bgl II is isolated from Bacillus globigii. One unit is the amount of enzyme required to cleave 1 µg of lambda DNA in one hour at 37°C in the appropriate buffer.

Components:

5213SA	Bgl II	Lot No.CCA101
Y90004	REact® 3	Lot No.CAV106

Storage Buffer:
50 mM Tris-HCl (pH 7.4)
150 mM KCl
1 mM Na_2EDTA
500 µg/ml BSA
50% (v/v) glycerol

Reaction Conditions:
Use REact® 3. REact® 3 is the 10X concentrate assay buffer supplied for use with Bgl II. Dilute 1 part in 10 in final reaction mixture.
Final concentration:
50 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$
100 mM NaCl
Assay at 37°C.
Store buffer at +4°C or -20°C.

Quality Control Data: See Back Page

Effective: 02/08/92

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OR THERAPEUTIC USE IN HUMANS OR ANIMALS.

GIBCO BRL
LIFE TECHNOLOGIES, INC.
GAITHERSBURG, MARYLAND 20898

5207SA
Lot No. CFF109
Hind III

5'-A↓AGCT T-3'
3'-T TCGA↑A-5'

2,000 units; 10 U/µl
Store at -20°C (not frost-free)

Description:
Hind III is isolated from Haemophilus influenzae R_d. One unit is the amount of enzyme required to cleave 1 µg of lambda DNA in one hour at 37°C in the appropriate buffer.

Components:

5207SA	Hind III	Lot No.CFF109
Y92500	REact® 2	Lot No.CE1106

Storage Buffer:
10 mM Tris-HCl (pH 7.4)
200 mM NaCl
0.5 mM Na_2EDTA
1 mM DTT
0.1% (w/v) TRITON® X-100
500 µg/ml BSA
50% (v/v) glycerol

Reaction Conditions:
Use REact® 2. REact® 2 is the 10X concentrate assay buffer supplied for use with Hind III. Dilute 1 part in 10 in final reaction mixture.
Final concentration:
50 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$
50 mM NaCl
Assay at 37°C.
Store buffer at +4°C or -20°C.

Quality Control Data: See Back Page

TRITON is a registered trademark of Rohm & Haas, Philadelphia, PA.

Effective: 02/08/92

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OR THERAPEUTIC USE IN HUMANS OR ANIMALS.

GIBCO BRL
LIFE TECHNOLOGIES, INC.
GAITHERSBURG, MARYLAND 20898

5201SA
BamH I

5'-G↓GATC C-3'
3'-C CTAG↑G-5'

Asm Lot # BH2103

PN #	LOT #
5201SA	BF0113
Y90004	BFR103

2,000 units; 10 U/µl
Store at -20°C (not frost-free)

BamH I is isolated from Bacillus amyloliquefaciens H. One unit is the amount of enzyme required to cleave 1 µg of lambda DNA in one hour at 37°C in the appropriate buffer.

Storage Buffer:
50 mM Tris-HCl (pH 7.5)
1 mM DTT
0.5 mM Na_2EGTA
300 mM KCl
500 µg/ml BSA
50% (v/v) glycerol

Reaction Conditions:
Use REact® 3. REact® 3 is the 10X concentrate assay buffer supplied for use with BamH I. Dilute 1 part in 10 in final reaction mixture.
Final concentration:
50 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$
100 mM NaCl
Assay at 37°C.
Store buffer at +4°C or -20°C.

Quality Control Data:
Endonuclease Assay: Cleavage only at recognition site at 100-fold excess digest of λ DNA.

Exonuclease Assay: Using 1 pmol radiolabeled termini in a 50 µl reaction and up to 20 units of enzyme for one hour, the following results were observed:
1 unit of enzyme removed ≤0.3% label from 5'ends.
1 unit of enzyme removed ≤0.3% label from 3'ends.

Ligation/Recut Assay: On λ DNA: ≥95% ligation in one hour at 22°C. 100% recut.
REFER TO THE BRL CATALOGUE AND REFERENCE GUIDE FOR NOTES ON CONDITIONS WHICH AFFECT ENZYME ACTIVITY.

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~~[illegible] mol volume of cold [illegible]~~

Digestion of pBlueBac III with 1) Bam HI and Bgl II
2) Bam HI and Hind III

1. Resuspend 10ug of pBlueBac III in ~~[illegible]~~ 100 ul of deionized H_2O
2. Set up the following digestions

1) pBlueBac III (5ug) - 50ul of 10ug/ml stock
10X React 3 buffer 10ul
~~Bam HI Bgl II~~ ok (10U/ul) 2.5ul
H_2O 35ul
TV = ~~100~~ 97.5 ul

Invitrogen 1-800-955-6288
Baculovirus Transfer
Vector pBlueBacIII
10 ug Lyophilized
20415 Store @: RT

2) pBlueBac III (5ug) - 50 ul of 10 ug/ml stock
10X React 2 buffer - 10ul
Hind III (10U/ul) 2.5ul
H_2O 35ul
TV = ~~100~~ 97.5 ul

10SEP92/ 11:20P Incubate @ 37°C for 2hrs. Then add:
2:20P 1) Bam HI, 2.5ul of 10U/ul

2) Bam HI, 2.5ul of 10U/ul
+
1ul of 5M NaCl (2.92g NaCl/10ml H_2O)

10Sep92/ 3:30P 2. ~~Extract the~~ Heat 65°C for ~~20~~ 5 min. Quench on ice ~~10~~ 5 min. Extract once w/ ø/$CHCl_3$. Back extract w/ 50ul 50mM Tris-HCl, pH8.0. Extract ~~once~~ again w/ ø/$CHCl_3$. Extract 2X w/ $CHCl_3$.

3. Precipitate the DNA w/ 1/10 volume 3M NaOAc, pH5.2, and 2 volumes 100% EtOH

11Sep92 Microfuge DNA for 30 min. @ 4°C. Wash the pellet w/ 100ul 70% EtOH. Freeze @ -70°C for 5 min. Microfuge for 5 min @ 4°C.

4. Resuspend DNA in 90ul of sterile H_2O and proceed to the alkaline phosphatase procedure. Add 1 ul to 0.8% LE agarose gel.

Removal of 5' phosphate from Bam HI/Bgl II and Hind III/Bam HI digested pBluebac III.

1. To 89ul of each pBBIII prep add:
10X CIP buffer 10ul
CIP (0.1U/ul) 1ul Dilute CIP 1:24 before use w/
100mM Tris, pH8.0 - 1ml of 1M
3M NaCl. - 1.75g
1mM $MgCl_2$ - 10ul of 1M
0.1mM $ZnCl_2$ - 10ul of 0.1M
QS to 10ml w/

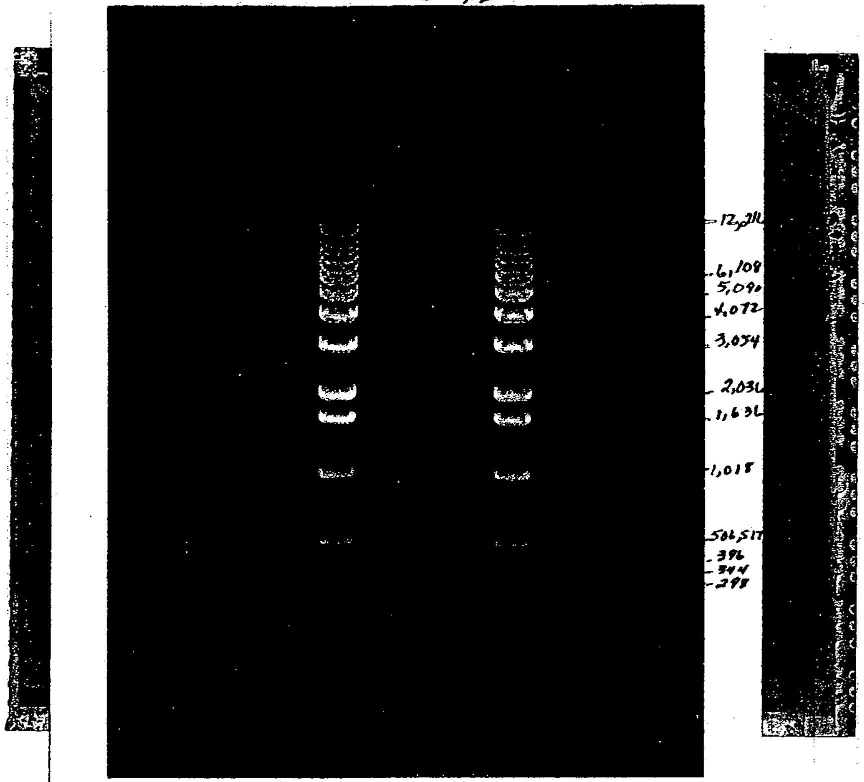
2. Incubate the reaction mixture at 37°C for 30 min.
3. Add 1 ul of 0.5 M EDTA to stop the reaction. (Forgot to add)
4. Heat the reaction to 75°C for 10 min to inactivate the CIAP.
5. Cool the tube to room temperature for 2 min.
6. Extract ~~[illegible]~~ with an equal volume of TE saturated phenol/chloroform. Back extract w/ 50 ul of 1X CIP buffer. Phenol/chloroform extract pooled aqueous solutions. Vortex 15 sec and microfuge 12,000 x g for 1 min for each extraction. Extract 2X w/ chloroform alone. Vortex 15 sec, microfuge 15 sec for each extraction.
7. Precipitate DNA w/ 1/10 volume 3M NaOAc, pH 5.2, and 2 volumes 100% EtOH.
8. Freeze on dry ice for 15 min or O/N @ -20°C.
9. Microfuge for 15 min at 4°C. Wash pellet w/ 100 ul of 75% EtOH. Place on dry ice or -70°C for 5 min. Microfuge 5 min @ 4°C. Dry pellet at edge of hood for 30 min @ R.T.
10. Quantitate entire sample at $A_{260/280}$ after resuspending DNA in 50 ul of H_2O. 1 A_{260} = 50 ug/ml.

```
      DUAL λ        02         Abs     Factor= 1.000
                                     Read average = 01
S#     260.0    280.0   λA/λB   S#    260.0    280.0   λA/λB
   50 ul sample  - pBBIII / R.E. cut / CIAP
1    -0.7070   4.6810 -.1510
2     0.0001   0.0003  .5000
3     1.1676   0.6268  1.862  BamHI 58.38 ug/ml = 0.05838 ug/ul = 2.92 ug in total sample
4     1.1540   0.6290  1.834  HindIII 57.1 ug/ml = 0.0571 ug/ul = 2.885 ug in total sample
                                                              5.805 ug - started w/
                                                              10 ug = ~58% recovery

==========================================================
  λ       ABS     SOURCE     14:47   CELL
260.0   1.1484   UV/Vis   09/14/92   sgle
```

11. Analyze 1 ul of each of the pBBIII preps on a 0.8% LE agarose gel in MPH rig using 1X TAE buffer.

Forgot to save uncut pBBIII to run on gel - major screwup



3,5

Ligation tube #	H_2O (ul)	5X ligase Buffer (ul)	pBB III Bgl II / Bam HI (ul) 0.~~[illegible]~~ 0.1 µg	pBB III Hind III Bam HI (ul) ~~0.[illegible]~~ 0.1 µg	T4 DNA ligase (ul)	Insert VEE 26S	VEE GP	SIN
1	~~4.25~~ 3	2	~~1.75~~ ~~6.0~~ 3.5		1	0.5	—	—
2	~~3.25~~ 2	2	~~1.75~~ ~~5.0~~ 3,5	—	1	~~2~~ 1	—	—
3	~~1.25~~ 0	2	~~1.75~~ 3,5	—	1	~~4~~ 2	—	—
4	~~4.25~~ 3	2	~~1.75~~ 3,5	—	1	—	~~1~~ 0,5	—
5	~~3.25~~ 2	2	~~1.75~~ 3,5	—	1	—	~~2~~ 1	—
6	~~1.25~~ 0	2	~~1.75~~ 3,5	—	1	—	~~4~~ 2	—
7	~~4.25~~ 3	2	— ~~1.75~~	~~1.75~~ 3,5	1	—	—	~~1~~ 0.5
8	3,25 2	2	— ~~1.75~~	~~1.75~~ 3,5	1	—	—	~~2~~ 1
9	~~1.25~~ 0	2	— ~~1.75~~	~~1.75~~ 3,5	1	—	—	~~4~~ 2
10	5.25	2	~~1.75~~ 3.5	—	1	—	—	—
11	5,25	2	—	~~1.75~~ 3,5	1	—	—	—

See next page for legible table

GIBCO BRL LIFE TECHNOLOGIES, INC.
GAITHERSBURG, MARYLAND 20898

5224SC
Lot No. CCK101
T4 DNA Ligase

500 units; 1 U/µl
Store at -20°C (not frost-free)

Description:
T4 DNA Ligase can be used to join DNA fragments with staggered or blunt ends and to repair nicks in double-stranded DNA having 3'hyd and 5'phosphate ends. The enzyme is isolated for *E. coli* lambda lyso NM989.

Unit Definition:
One (Weiss) unit catalyzes the exchange of 1 nmol ^{32}PPi into [γ/β-^{32}P]ATP in 20 minutes at 37°C.[1]

Components:

5224SC	T4 DNA Ligase	Lot No. CCA107
Y90001	5X DNA Ligase Buffer	Lot No. CCA106

Storage Buffer:	5X DNA Ligase Reaction Buffer:
10 mM Tris-HCl (pH 7.5)	250 mM Tris-HCl (pH7.6)
50 mM KCl	50 mM $MgCl_2$
1 mM DTT	5 mM ATP
50% (v/v) glycerol	5 mM DTT
	25% (w/v) polyethylene glycol-8000.

Store buffer at -20°. Due to the presence of dilute ATP, the shelf life of the buffer is 6 months.

Note:
FOR OPTIMAL TRANSFORMATION, DILUTE THE LIGATION REACTION ≥ 5-FOLD PRIOR TO ADDING TO COMPETENT CELLS[2].

Quality Control Data: See Back Page

Effective: 02/00/92

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```
     SINGLE λ       02        Abs
                                   Read average = 01
S#   600.0          S#   600.0          S#   600.0

1    0.0000
2    0.1887  TOP10 cells 11:32A









======================================================
  λ        ABS      SOURCE     11:33    CELL
600.0    0.0002   Visible 09/17/92    1
```

```
     SINGLE λ       02        Abs
                                   Read average =
S#   600.0          S#   600.0          S#   600.

3    0.0007
4    0.9647  TOP10 cells 1:08P.









======================================================
  λ        ABS      SOURCE     13:08    CELL
00.0     0.0008   Visible 09/17/92    1
```

0.05 · 10 = x
x = 0.5 ml

0.05 · [illegible] = x
x = 0.25

0.05 · 20 = x

	Ligation#	H_2O	5X ligase buffer	pBBIII BglII BamHI	pBBIII HindIII BamHI	Insert VEE 26S	VEE GP	SIN	T4DNA LIGASE
15SEP92	1	3	2	3.5 ✓	—	0.5 ✓	—	—	1
3:30P	2	2.5	2	3.5 ✓	—	1 ✓	—	—	1
	3	1.5	2	3.5 ✓	—	2 ✓	—	—	1
	4	3	2	3.5 ✓	—	—	0.5 ✓	—	1
	5	2.5	2	3.5 ✓	—	—	1 ✓	—	1
	6	1.5	2	3.5 ✓	—	—	2 ✓	—	1
	7	3	2	—	3.5 ✓	—	—	0.5 ✓	1
	8	2.5	2	—	3.5 ✓	—	—	1 ✓	1
	9	1.5	2	—	3.5	—	—	2 ✓	1
	10	3.5	2	3.5 ✓	—	—	—	—	1
	11	3.5	2	—	3.5 ✓	—	—	—	1

Incubate ~~ON~~ 2D at 16°C

15SEP92 Take the TOP 10 stab provided and streak out a small portion on an LB media plate without ampicillin and incubate @ 37°C ON.

16SEP92 Pick a single colony and transfer it into 2 ml of SOB media in a Falcon 2059 tube ~~[illegible]~~ Incubate ON @ 37°C at 200 cycles/min in a rotary shaker.

17SEP92 10A Transfer 2 ml culture to 100 ml of SOB (prewarmed) media in a 1L 500 ml screw cap Erlenmeyer flask. Incubate for approximatly 3 hrs @ 37°C with vigorous shaking (200 cycles/m Monitor growth by determining the OD at A_{600} ~~260~~.

2. When the OD at A_{600} ~~260~~ of the culture reaches approximately 0.5, collect the cells by centrifugation (4000 rpm, 4°C, 10 min in a Sorvall GS4 rotor) and resuspend the pellet in

→ 10 ml ice-cold 50 mM $CaCl_2$. Incubate cells on ice for at least 30 min. It is easier to resuspend the pellets if they are vortexed before $CaCl_2$ is added as some clumping can occur. The cells can be uniformly suspended by aspirating up and down with a 25 ml pipet.

→ 3. Centrifuge $CaCl_2$-treated cells in a 4°C rotor (4000 rpm, 4°C, 5 min, Sorval ~~SS34~~ GSA rotor). NO BRAKE Gently resuspend the cells in 4 ml of ice-cold 50 mM $CaCl_2$. Keep the cells on ice.

4. Aliquot 100 ul of $CaCl_2$-treated cells for each transformation into a pre-chilled (on ice) Falcon 2059 tube. Add transforming DNA - 5 ul of each ligation mixture (10-100 ng) and incubate on ice for 30 min.

17Sep92 2:23P 5. After 30 min. on ice, heat shock cells at 42°C for 45 sec (in a water bath). After heat shock treatment, return the tubes to ice for and additional 2 min

6. Preheat appropriate number of LB plts w/ampicillin to 37°C

Grid plates - 1-50 = VEE 2
51-100 = VEE G
101-150 = SIN
151-160 = #10 =
161-170 = #11

```
         SINGLE λ          02          Abs
                                                Read average = 01
 S#    600.0               S#    600.0                S#    600.0

 1     -0.0001
 2      0.7031
```

```
   λ          ABS        SOURCE      11:04     CELL
 600.0      -0.0001     Visible 09/23/92        1
```

```
        SINGLE λ          02          Abs
                                               Read average = 01
 S#    600.0              S#    600.0                S#    600.0

 3     0.0011
 4     0.5460
```

added 40 ml of SOB media to the original 100ml.

```
   λ          ABS        SOURCE      11:16     CELL
 600.0       0.0011     Visible 09/23/92        1
```

7. Add 1 ml SOC media (BRL Cat# 5544SA) and incubate the cultures for 45 min at 37°C with vigorous shaking (200 cycles/min) in a rotary shaker.
8. Plate 100 ul of cells onto LB plates containing 50 ug/ml ampicillin. Use glass spreaders to provide even "lawn" of cells.

18 SEP 92 Plate out 50 colonies from each of VEE 26S, VEE CP, and S1N transformation onto grid plates. Touch a colony with a sterile toothpick, streak the colony onto a grid plate section and then place the toothpick into 2 ml LB broth containing 50 ug/ml ampicillin. Incubate the liquid cultures ON ~~at~~ 37°C in rotary shaker (200 cycles/min). Incubate grid plate in walk-in incubator @ 37°C.

~~19 Sep 92 Place all tubes at 4°C~~

19 Sep 92 11:00A Grid plates grew colonies. (Colonies seemed larger than normal however.) Broth ~~plates~~ tubes were clear. When spun at 10,000xg for 10 min, no pellet was seen. Grid plates were placed at 4°C.

21 Sep 92 No growth in broth tubes. Discarded.
Made up new LB broth and new LB media.

22 SEP 92 No growth on newly streaked LB plts + Amp with ligations 15 Sep 92. No growth in media inoculated for minipreps from grid plts.

22 SEP 92

Ligation	H_2O	5X ligase buffer	pBBIII BglII BamHI	~~pBBIII HindIII BamHI~~	Inserts: VEE 26S	VEE CP	BglII BamHI 26 WAT #11 VC-C1 VC CAPS; B'
1	3	2	3.5		0.5	—	—
2	2.5	2	3.5		1	—	—
3	1.5	2	3.5		2	—	—
4	3	2	3.5		—	0.5	—
5	2.5	2	3.5		—	1	—
6	1.5	2	3.5		—	2	—
7	2.5	2	3.5		—	—	1
8	3.5	2	3.5		—	—	—

Incubate ON @ 16°C.

23 Sep 92 8:45A Transfer 2 ml culture to 100 ml of SOB (prewarmed) media ~~in~~ a 1 L screw cap Erlenmeyer flask. Incubate for approximately 2 H @ 37°C with shaking (200 cycles/min.)
Monitor growth by determining the OD @ A_{600}.

20 0.07 1.17 1 ml
2. When the OD at A_{600} of the culture reaches approximately 0.5, collect the cells by centrifugation (4000 rpm, 4°C, 10 min in a Sorvall GSA rotor) and resuspend the pellet in 10 ml ice cold 50 mM CaCl. Incubate the cells on ice for at least 30 min. It is easier to resuspend the pellets if they are vortexed before CaCl₂ is added as some clumping can occur. The cells can be uniformly suspended by aspirating up and down with a 25 ml pipet

Have 100 mg AMP in 5 ml H_2O =
20 ug/ul

Have ~ 50 colonies -
Need 100 ml broth
Need 5000 ug of ampicillin
Add 250 ul of stock AMP
to 100 ml LB broth

3. Centrifuge $CaCl_2$-treated cells in a 4°C rotor (4000 rpm, 4°C, 5 min, Sorvall ~~[illegible]~~ SS-34 rotor). Gently resuspend the cells in 4 ml of ice-cold 50 mM $CaCl_2$. Keep the cells on ice.
4. Aliquot 100 ul of $CaCl_2$-treated cells for each transformation into a pre-chilled (on ice) Falcon 2059 tube. Add transforming DNA - 5 ul of each ligation mixture (10-100 ng) and incubate on ice for 30 min.

1:40P 5. After 30 min on ice, heat shock cells @ 42°C for 45 sec in a water bath. After heat shock treatment, return the tubes to ice for an additional 2 min.
6. Preheat appropriate number of LB w/ampicillin to 37°C
7. Add 1 ml SOC media (BRL Cat#) and incubate the cultures for 45 min @ 37°C with vigorous shaking (200 cycles/min) in a rotary shaker.
8. Plate 100 ul of cells onto LB plates containing 50 ug/ml ampicillin. Use glass spreaders to provide even lawn of cells.

24 Sep 92 1.

	Plate #	# of colonies	Grid plt #	Replate of transform.	
Ligation of 23SEP	1	5	1-5	✓	
	2	2	6-7	✓	150 ul of ea
	3	3	8-10	✓	
	4	1	11	✓	
	5	5	12-16	✓	
	6	8	17-24	✓	
	7	6	26-31		200 ul of Lig 1-6 O.N.
	8	4	32-35		
Ligation of 19 Sep 92	7	1	36	✓	29 Sep 92
	8	0	—		
	9	2	37-38	✓	
	11	2	39-40		
	Cells only	0	—		

Transferred all colonies to grid plates (LB agar + ampicillin). Touch a colony with a sterile toothpick, streak the colony onto a grid plate section and then place the toothpick into 2 ml LB broth containing 50 ug/ml ampicillin. Incubate the liquid culture ON @ 37°C in rotary shaker (200 cycles/min.) - Incubate grid plates in walk in incubator @ 37°C.

Minipreps of Colonies 1-40

Procedure:

1. Pellet 1.5 ml ON culture for 10 min.

150
100
250

2. Resuspend pellet in 100 ul 50 mM Tris, pH 8.0, 50 mM EDTA containing lysozyme - used an amount equal to ~~a~~ 1/4 [illegible])

GIBCO BRL

LIFE TECHNOLOGIES, INC.
GAITHERSBURG, MARYLAND 20898

5213SB
Lot No. CCS103
Bgl II

5'-A↓GATC T-3'
3'-T CTAG↑A-5'

2,000 units; 10 U/µl
Store at -20°C (not frost-free)

Description:
Bgl II is isolated from Bacillus globigii. One unit is the amount of enzyme required to cleave 1 µg of lambda DNA in one hour at 37°C in the appropriate buffer.

Components:

5213SB	Bgl II	Lot No.CCS103
Y90004	REact® 3	Lot No.BH9107

Storage Buffer:
50 mM Tris-HCl
(pH 7.4)
150 mM KCl
1 mM Na_2EDTA
500 µg/ml BSA
50% (v/v) glycerol

Reaction Conditions:
Use REact® 3. REact® 3 is the 10X concentrate assay buffer supplied for use with Bgl II. Dilute 1 part in 10 in final reaction mixture.
Final concentration:
50 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$
100 mM NaCl
Assay at 37°C.
Store buffer at +4°C or -20°C.

Quality Control Data: See Back Page

Effective: 02/00/92

FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC
OR THERAPEUTIC USE IN HUMANS OR ANIMALS.

3. Vortex.
4. Add 200ul 1% SDS /200 mM NaOH, mix by inversion and incubate at RT for 5 min.
5. Add 150ul 3M KOAc.
6. Mix gently by inversion (DO NOT OVERMIX). Incubate on ice for 3-5 min.
7. Pellet 5 min.
8. Transfer supernatant to clean tube. Add 1 ml isopropanol
9. Mix and pellet 10 min.
10. Wash pellet w/ 100ul 75% ETOH. and speed vac to dryness.
11. Add 50ul of H_2O and 0.5ul BM RNase, DNase free. Incubate 30 min @ 37°C

Restriction endonuclease analysis:

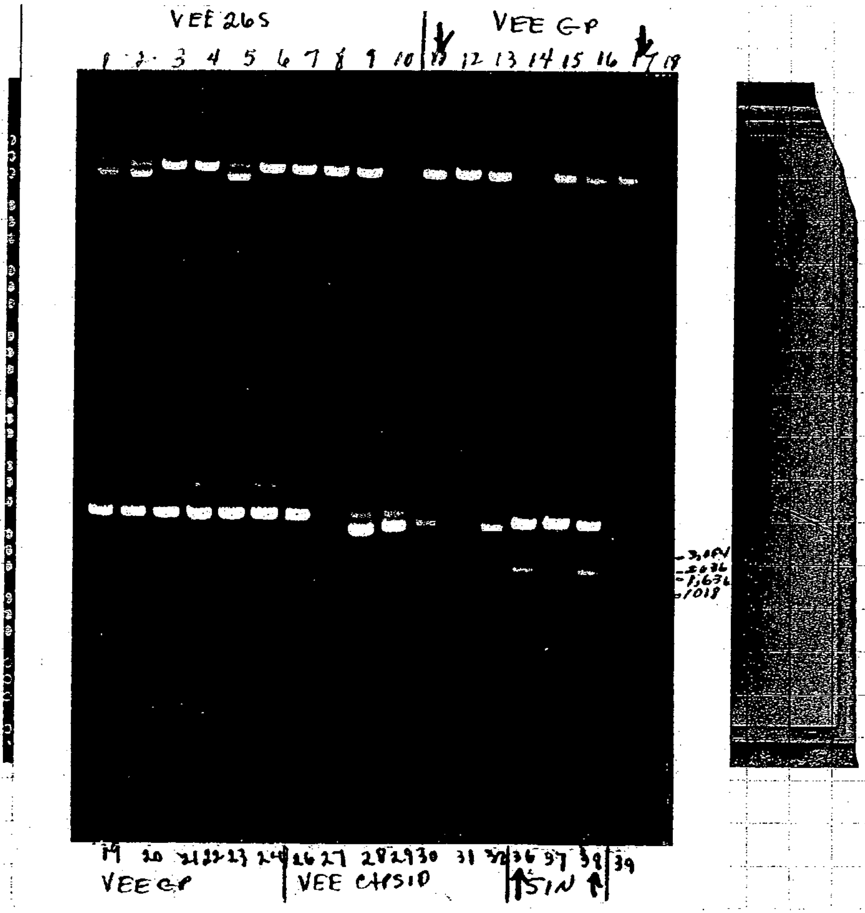
Colony#	Ligation #	Date	Insert	Bgl II / BamHI (React 3)	React 2 / 0.1ul 5M Hind III / BamHI
1	1 -	23 Sep	VEE 26S	✓	
2	1	"	"	✓	
3	1	"	"	✓	
4	1	"	"	✓	
5	1	"	"	✓	
6	2	"		✓	
7	2	"	VEE GP	✓	
8	3	"	"	✓	
9	3	"	"	✓	
10	3	"	"	✓	
11	4	"	"	✓	
12	5	"	"	✓	
13	5	"	"	✓	
14	5	"	"	✓	
15	5	"	"	✓	
16	5	"	"	✓	
17	6	"	"	✓	
18	6	"	"	✓	
19	6	"	"	✓	
20	6	"	"	✓	
21	6	"	"	✓	
22	6	"	"	✓	
23	6	"	"	✓	
24	6	"	"	✓	
~~25~~					
26	7	"	VEE CAPSID	✓	
27	7	"	"	✓	
28	7	"	"	✓	
29	7	"	"	✓	

Colony#	Ligation #	Insert	Bgl II / Bam HI (React 3)	React 2 / 0.1 ul NaCl (5M) Hind III / Bam HI
30	7-23 Sep	VEE CAPSID	✓	
31	7 "	"	✓	
32	7 "	"	✓	
~~33~~				
~~34~~				
~~35~~				
36	7 15 Sep	SIN		✓
37	9 "	"		✓
38	9 "	"		✓
39	11 "	NO INSERT		✓

For each restriction analysis:

~~#1~~ 12 ul H_2O
2 ul 10X Buffer (React 3 buffer for #1-32, React 2 for tubes 36-39)
5 ul DNA
1 ul RE
TV = 20 ul

The Bgl II or HIND III restriction enzyme was added to respective tubes, incubate 1 H @ 37°C. after 1 H, Bam HI was added to tubes 1-32 and 0.1 ul of 5M NaCl and 1 ul of Bam HI was added to tubes 36-39.



No inserts of VEE 26S or VEE GP's were found after analysis. 150 ul of ligation 1-6 of 23 Sep were spread onto LB w/AMP and colonies allowed to grow ON @ 37°C.

Ligation	Insert	# of COLONIES	DESIGNATION
1	VEE 26S	4	41-44
2	"	2	45-46
3	"	4	47-50
4	VEE GP	23	51-73
5	"	27	74-100
6	"	20	101-120

Colonies 1, 2, 5, 10, 14, 18, 27-32 were reanalyzed.

30 Sep 92 **Minipreps**: See procedure p 52-53 of this notebook.

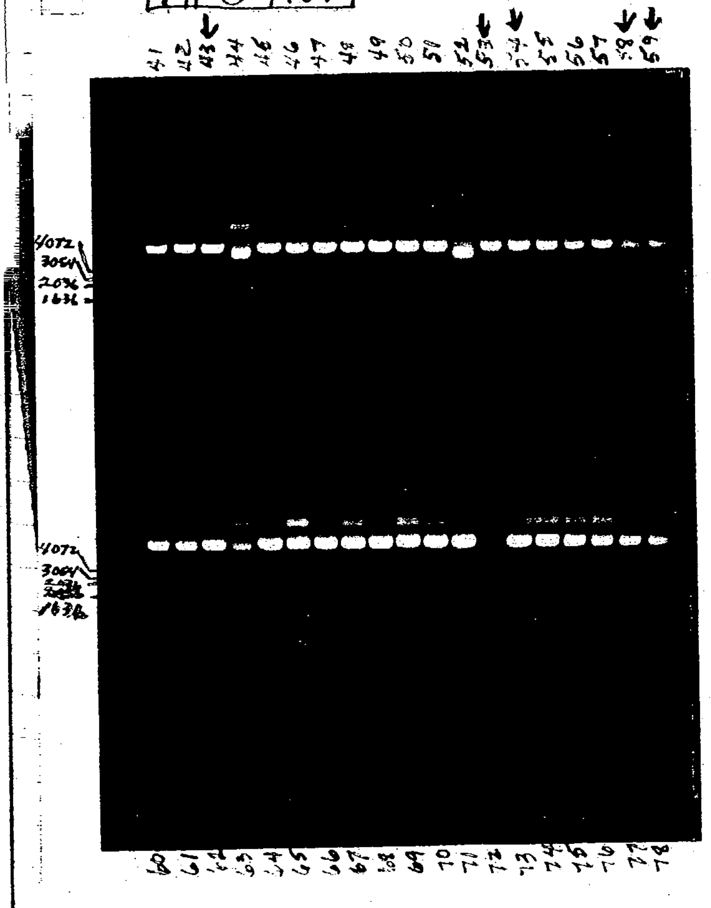
R.E. analysis: Have 40 minipreps (#41-80)

Make reagents for 45.

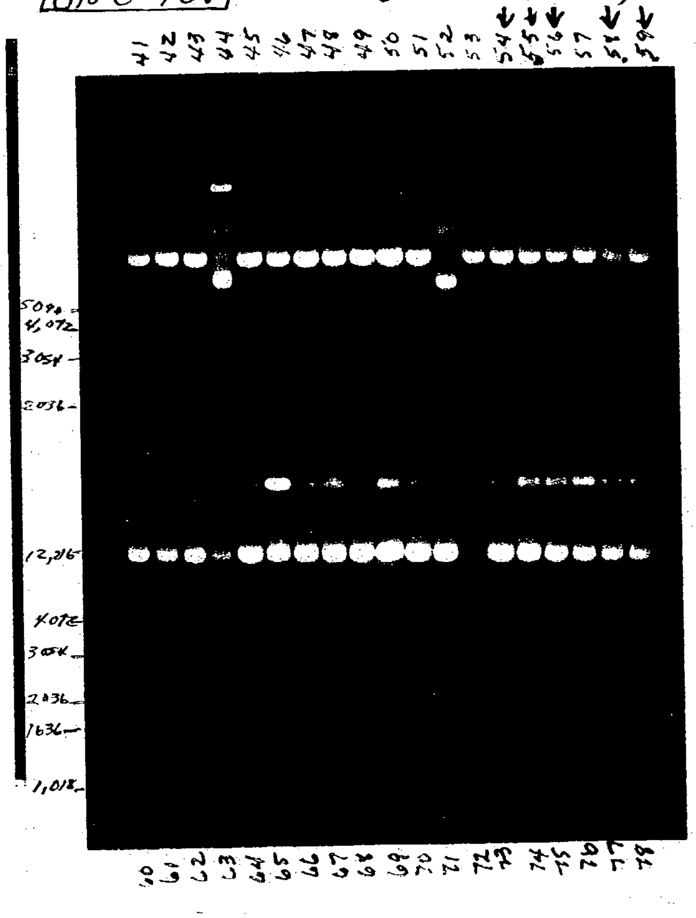
1X
1 ul Bam HI
1 ul Bgl II
2 ul React 3 buffer
3 ul miniprep DNA
13 ul H2O
TV = 20 ul

MM:
45 ul Bam HI
45 ul Bgl II
90 ul React 3
585 ul H2O
TV = 765 ul
135 ul DNA
900 ul
Add 17 ul / tube.

1H @ 190V

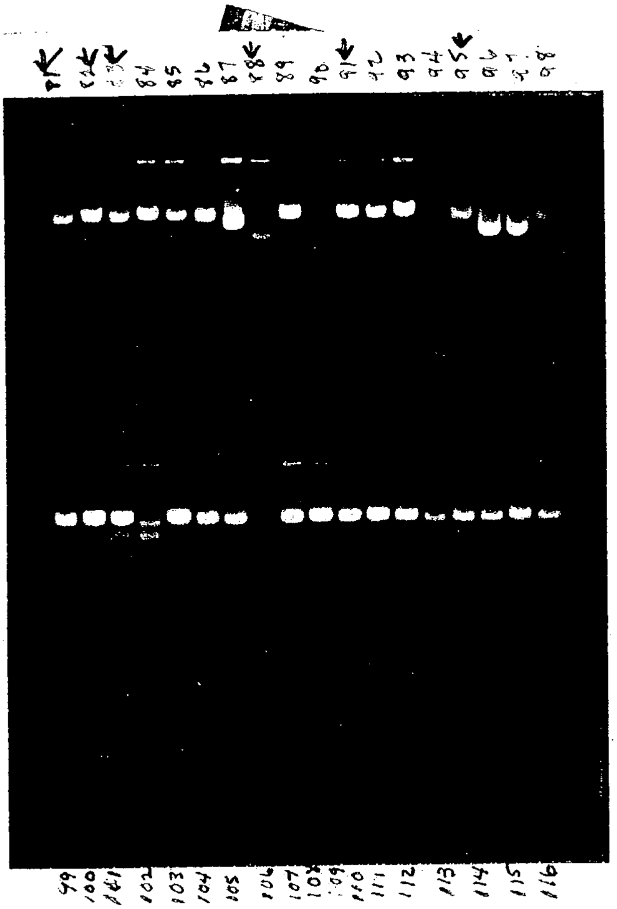


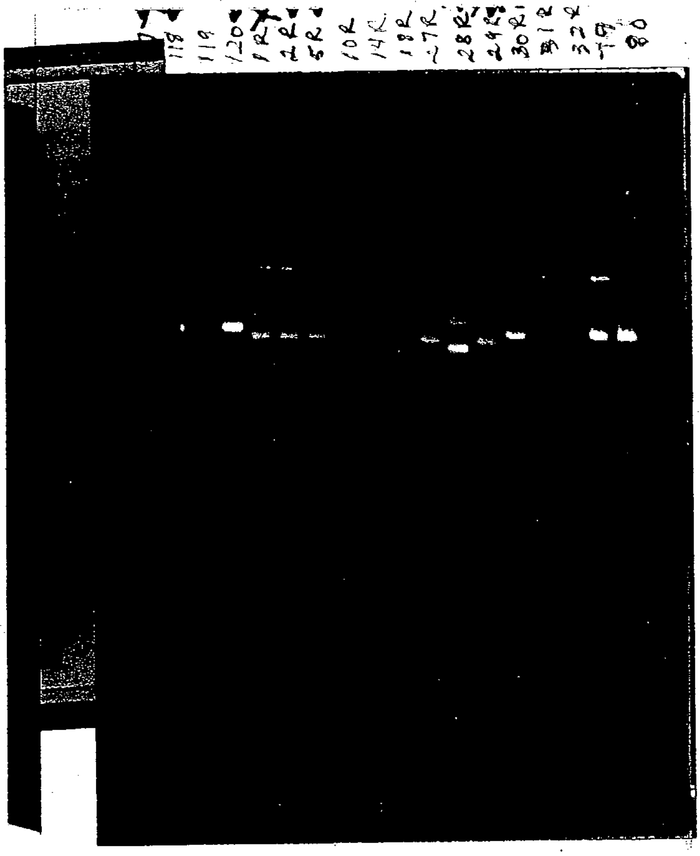
ON @ 40V



1Oct92 RE Analysis of Minipreps 81-120 and 1, 2, 5, 10, 14, 18, 27-32 repeats.
Have 52 minipreps (make reagents enough for 55)

MM: 55 ul BamHI
55 ul BglII
110 ul React 3
115 ul H_2O





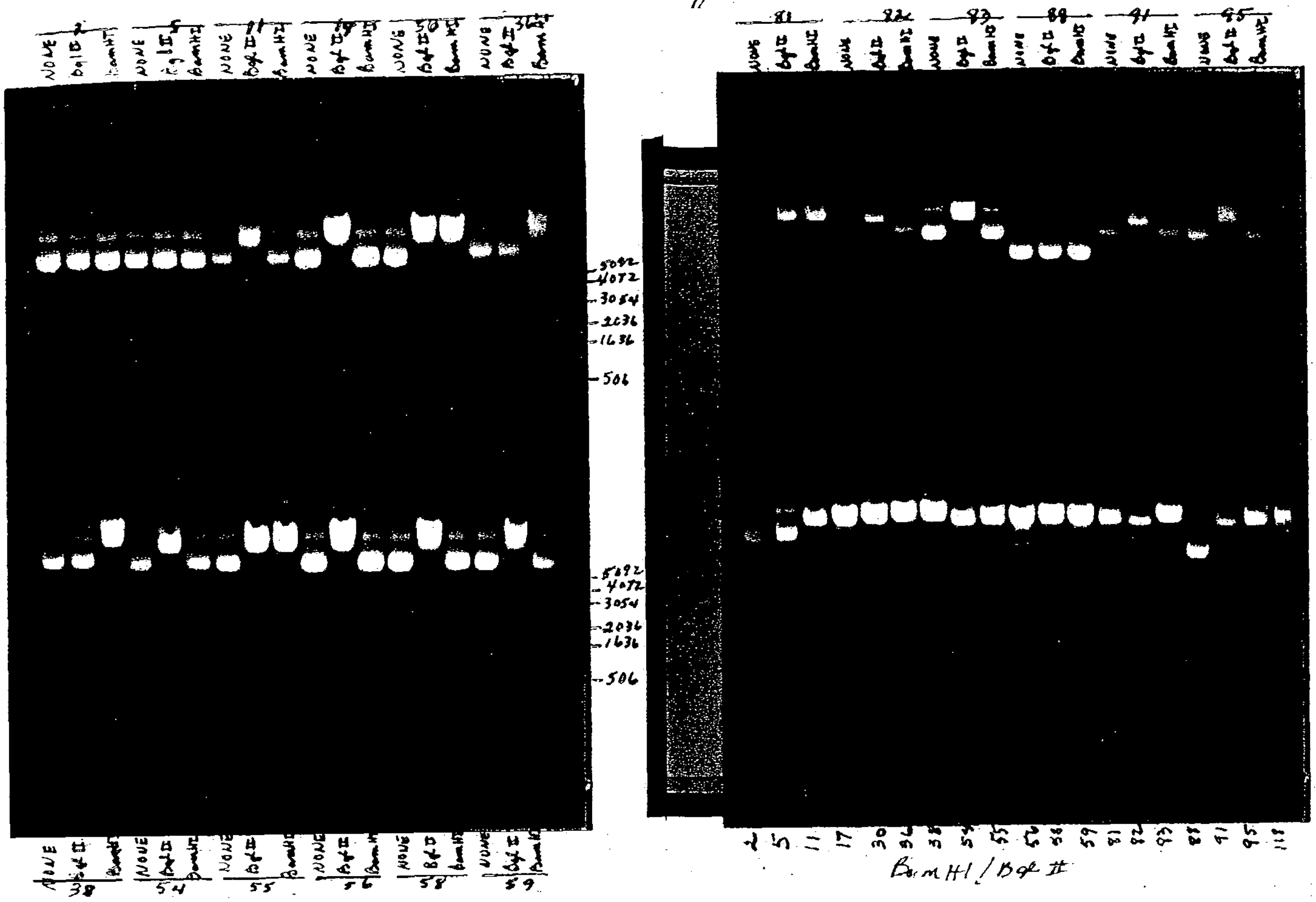
1Oct92 Following colonies were selected for further RE analysis
2, 5, 11, 14, 30, 36, 38, 54, 55, 56, 58, 59, 81, 82, 83, 88, 91, 95,
118, 120.
Have 20 colony picks.

2Oct92 Minipreps - Promega method.

4 reactions: w/ BamHI
w/ BglII
w/ BamHI/BglII
no enzyme

MM BamHI	MM BglII	MM BamHI/BglII	No enzyme
25 ul BamHI	25 ul BglII	12.5 ul BamHI	50 ul React3
50 ul React3	50 ul React3	12.5 ul BglII	200 ul H_2O
175 ul H_2O	175 ul H_2O	50 ul React3	
		175 ul H_2O	

Incubated w/ BamHI, w/ Bgl II, no enzyme controls for 1 H @ 37° (individually set up)
Incubate w/ BamHI/ Bgl II tubes for 2H @ 37°C.



Colony#	Bgl II	BamHI	INSERT
2	–	–	VEE26S
5	–	–	"
11	+	–	VEEGP
17	+	–	"
30	+	+	VEE CAPSID
36	–	+	SIN
38	–	+	SIN
54	+	–	VEE GP
55	+	+	↓
56	+	–	
58	+	–	
59	+	–	
81	+	+	
82	+	–	
83	+	–	
88	+	–	
91	–	–	
95	+	–	

Colonies 118 and 120 were analyzed but not run on gels.

SIN, SIN } no Bgl II site - has Hind III site.

BRL Bethesda Research Laboratories
Life Technologies, Inc.
Gaithersburg, MD 20877 USA

5213SA
Lot # BCU401
Bgl II

5'-A↓GATC T-3'
3'-T CTAG↑A-5'

400 units; 10 U/µl
Store at -20°C (not frost-free)

Bgl II is isolated from Bacillus globigii. One unit is the amount of enzyme required to cleave 1 µg of lambda DNA in one hour at 37°C in the appropriate buffer.

Storage Buffer:
50 mM Tris-HCl
(pH 7.4)
150 mM KCl
1 mM Na_2EDTA
500 µg/ml BSA
50% (v/v) glycerol

Reaction Conditions:
Use REact® 3. REact® 3 is the 10X concentrate assay buffer supplied for use with Bgl II. Dilute 1 part in 10 in final reaction mixture.
Final concentration:
50 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$
100 mM NaCl
Assay at 37°C.
Store buffer at +4°C or -20°C.

Quality Control Data:
Endonuclease Assay: Cleavage only at recognition site at 100-fold excess digest of λ DNA.

Exonuclease Assay: Using 1 pmol radiolabeled termini in a 50 µl reaction and up to 20 units of enzyme for one hour, the following results were observed:
1 unit of enzyme removed ≤0.3% label from 5'ends.
1 unit of enzyme removed ≤0.3% label from 3'ends.

Ligation/Recut Assay: On λ DNA: >95% ligation one hour at 22°C. 100% recut.
REFER TO THE BRL CATALOGUE AND REFERENCE GUIDE FOR NOTES ON CONDITIONS WHICH AFFECT ENZYME ACTIVITY.

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GIBCO BRL
LIFE TECHNOLOGIES, INC.
GAITHERSBURG, MARYLAND 20898

5201SA
Lot No. CCU105
BamH I

5'G↓GATC C-3'
3'C CTAG↑G-5'

2,000 units; 10 U/µl
Store at -20°C (not frost-free)

Description:
BamH I is isolated from Bacillus amyloliquefaciens H. One unit is the amount of enzyme required to cleave 1 µg of lambda DNA in one hour at 37°C in the appropriate buffer.

Components:

5201SA	BamH I	Lot No. CCU105
Y90004	REact® 3	Lot No. CAV107

Storage Buffer:
50 mM Tris-HCl (pH 7.5)
1 mM DTT
0.5 mM Na_2EGTA
300 mM KCl
500 µg/ml BSA
50% (v/v) glycerol
0.1% (w/v) Triton X-100

Reaction Conditions:
Use REact® 3. REact® 3 is the 10X concentrate assay buffer supplied for use with BamH I. Dilute 1 part in 10 in final reaction mixture.
Final concentration:
50 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$
100 mM NaCl
Assay at 37°C.
Store buffer at +4°C or -20°C.

Quality Control Data: See Back Page

Triton® is a registered trademark of Rohm & Haas, Philadelphia, PA.

Effective: 02/07/92

ct-92
0A

Maxipreps

Selected #30 (VEE CAPSID), #55 (VEEGP), #81 (VEEGP), #36+38 (SIN) ~~for~~ and #39 for repeat of minipreps and maxipreps.

Miniprep and RE analysis

athy
id these
see her
book
p 26-31
Notebook #346

Minipreps: Promega

RE: Colony picks **#30, 55, 81 and 39**:

1X: 0.5 ul Bam HI
0.5 ul Bgl II
2 ul React 3
3 ul Miniprep DNA
14 ul H_2O

Master Mix:
17ul MM
70 ul H_2O
10 ul React 3 buffer
2.5 ul Bam HI
2.5 ul Bgl II
TV = 85 ul

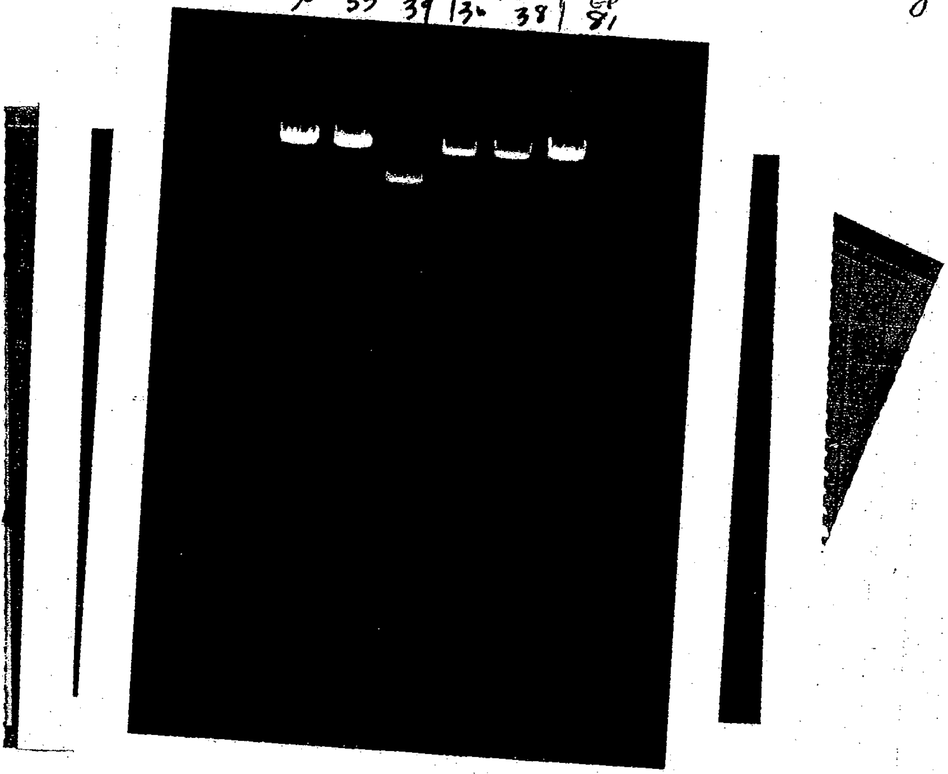
Incubate 1H @ 37°C
Analyze on MPH, 1% agarose gel, w/EtBr in gel

RE: Colony picks #36 and 38

1X: 1 ul Hind III
2 ul React 2
3 ul Miniprep DNA
14 ul H_2O

Master mix
17ul MM
42 ul H_2O
6 ul React 2 buffer
3 ul Hind III

Incubate 1H @ 37°C.
Add: 1 ul of Bam HI and 0.1 ul of 5M NaCl to each tube. Incubate 1H @ 37°C
Analyse on MPH 1% agarose gel w/ EtBr in gel.



```
        SINGLE λ      02      Abs
                                  Read average = 01
S#   600.0        S#   600.0        S#   600.0

1   -0.0001
2    0.1454 36
3    0.1460 38
4    0.7534 39

===============================================
 λ       ABS     SOURCE    14:38    CELL
600.0  -0.0602   Vis/UV  10/06/92    1
```

```
        SINGLE λ          02         Abs
                                          Read average = 01
S#     600.0              S#    600.0          S#    600.0

5      0.0020
6      0.2063 36
7      0.1986 38
8     -0.0591

=====================================================================
 λ            ABS        SOURCE      15:38     CELL
600.0        0.0020      Vis/UV    10/06/92     1
```

```
        SINGLE λ          02         Abs
                                          Read average = 01
S#     600.0              S#    600.0          S#    600.0

9      0.0044
10     0.3201 36
11     0.2880 38
12    -0.0579

=====================================================================
 λ            ABS        SOURCE      16:17     CELL
```

Maxipreps of 36 & 38 (SIN CP's) and 39 (pBBIII).

5Oct92 Made 2 LB/AMP tubes for each of the colonies to be analyzed. Cathy Schmidt did minipreps on 1 set of the LB/AMP ~~tubes~~ cultures, the remaining set ~~wo~~ continued to incubate @ 37°C in shaker. At ~11AM the 2 ml broths were added to 25 ml of LB/AMP.

5Oct92 2:30P Aliquots of the LB/AMP broths were used to determine OD of 25 ml LB/AMP preps. see ~~readings~~ readings on plot-top printout. Prep #39 was added to 500 ml of LB/AMP broth and incubated ON @ 37°C.

3:30P 2 more readings were taken of the other 2 cultures.

4:20P #36 growth the fastest and was selected to maxiprep.

```
                 DUAL λ        01         Abs      Factor= 1.000
                                                   Read average = 01
             S#     260.0    280.0    λA/λB    S#    260.0      280.0    λA/λB
                    10ul in 500ul : 1:51 dil         50ug/ml    500ul sample
             7      0.0000   -0.0001  -0.000
SIN     36   8      2.2052    1.2190   1.809   5,623.26 ug/ml  = 2.8 mg total sample
pBBIII  39   9      1.3320    0.7330   1.817   3,396.6 ug/ml   = 1.7 mg total sample
                 SIN = 5.6 ug/ul
                 pBBIII = 3.4 ug/ul



            ==============================================================
               λ         ABS       SOURCE     14:23     CELL
            260.0     -0.0444     UV/Vis    10/08/92     1
```

9oct92 ~~Maxipreps~~ Minipreps of CCHF & VEE HIS NC Preps and SIN-GP and pBBIII.

~~Maxipreps~~ Minipreps and glycerol stocks were made of CCHF C-29 and VEE-1 from LB/AMP grid plts. 2 ~~sets~~ of 2ml LB broths were set up on each colony. 2 sets of 2ml LB broth were also set up on SINGP.

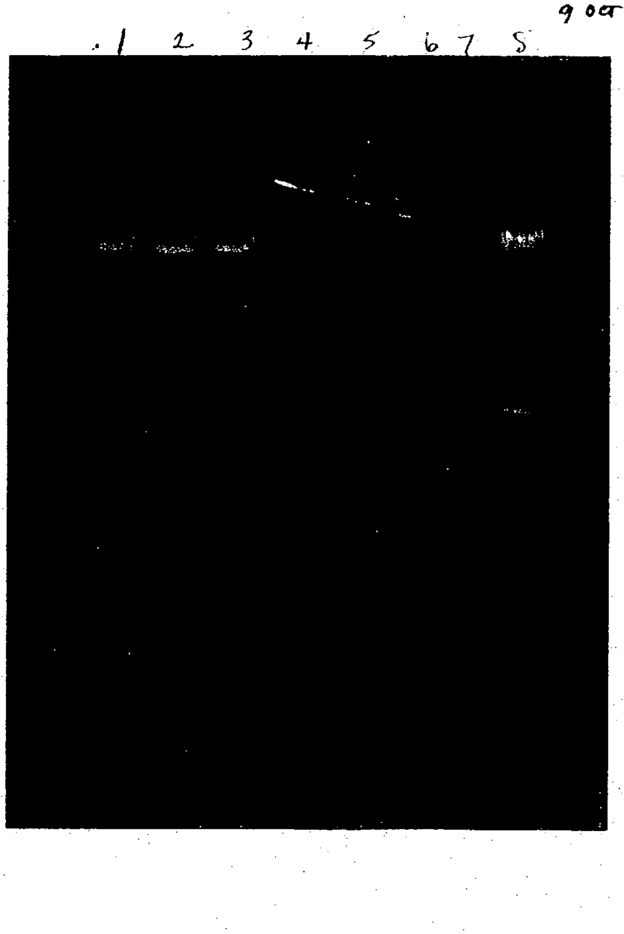
Minipreps were done by Cathy Schmidt, see notebook 3460 p33.

Restriction endonuclease analysis:

pBBHIS/CCHF-NC
pBBHIS/VEE/NC }
12ul H_2O
2ul React 3 buffer
5ul miniprep DNA
0.5ul Bam HI
0.5ul Bgl II
Inc @ 2H @ 37°C. Ice 10 min.

pBBIII/SINGP:
12ul H_2O
2ul React 2 buffer
5ul miniprep DNA
1 ul HIND III
Inc 1 H @ 37°C, then add:
1ul Bam HI + 0.1ul 5M NaCl
Inc 1H @ 37°C. Ice 10 min.

9oct92 Analyze on 1% LE (Seakem) agarose gel:



9 oct 92

minipreps {
1 - pBBHIS/CCHF-NC
2 - pBB III/SIN GP
3 - pBBHIS/VEE-NC
4 - #39 pBBIII - UNCUT
5 - #39 pBBIII BamHI
6 - #39 pBBIII BglII
7 - #39 pBBIII HindIII
8 - pBBIII/SINGP

GIBCO BRL LIFE TECHNOLOGIES, INC.
GAITHERSBURG, MARYLAND 20898

5215SB
Lot No. CE5108
Pst I

5'-C TGCA↓G-3'
3'-G↑ACGT C-5'

1,000 units; 10 U/µl
Store at -20°C (not frost-free)

Description:
Pst I is isolated from Providencia stuartii. One unit is the amount of enzyme required to cleave 1 µg of lambda DNA in one hour at 37°C in the appropriate buffer.

Components:

5215SB	Pst I	Lot No.CE5108
Y92500	REact® 2	Lot No.CAV105

Storage Buffer:
10 mM Tris-HCl
(pH 7.4)
50 mM NaCl
0.1 mM Na_2EDTA
1 mM DTT
0.15% (w/v) Triton X-100
50% (v/v) glycerol

Reaction Conditions:
Use REact® 2. REact® 2 is the 10X concentrate assay buffer supplied for use with Pst I. Dilute 1 part in 10 in final reaction mixture.
Final concentration:
50 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$
50 mM NaCl
Assay at 37°C.
Store buffer at +4°C or -20°C.

Triton® is a registered trademark of Rohm & Haas, Philadephia, PA.

Quality Control Data: See Back Page

Effective 02/00/92

FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE IN HUMANS OR ANIMALS.

BRL Bethesda Research Laboratories
Life Technologies, Inc.
Gaithersburg, MD 20877 USA

5421SC
Lot # BD4406
Nco I

5'-C↓CATG G-3'
3'-G GTAC↑C-5'

200 units; 10 U/µl
Store at -20°C (not frost-free)

Nco I is isolated from Rhodococcus sp ATCC 19070. One unit is the amount of enzyme required to cleave 1 µg of lambda DNA in one hour at 37°C in the appropriate buffer.

Storage Buffer:
10 mM Tris-HCl
(pH 7.5)
50 mM KCl
0.1 mM Na_2EDTA
1 mM DTT
0.1% (w/v) Triton-X-100
100 µg/ml BSA
50%(v/v) glycerol

Reaction Conditions:
Use REact® 3. REact® 3 is the 10X concentrate assay buffer supplied for use with Nco I. Dilute 1 part in 10 in final reaction mixture.
Final concentration:
50 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$
100 mM NaCl
Assay at 37°C.
Store buffer at +4°C or -20°C.

Quality Control Data:
Endonuclease Assay: Cleavage only at recognition site at 100-fold excess digest of λ DNA.

Exonuclease Assay: Using 1 pmol radiolabeled termini in a 50 µl reaction and up to 20 units of enzyme for one hour, the following results were observed:
1 unit of enzyme removed ≤0.3% label from 5'ends.
1 unit of enzyme removed ≤0.3% label from 3'ends.

Ligation/Recut Assay: On λ DNA: ≥95% ligation in one hour at 22°C. 100% recut.
REFER TO THE BRL CATALOGUE AND REFERENCE GUIDE FOR NOTES ON CONDITIONS WHICH AFFECT ENZYME ACTIVITY.

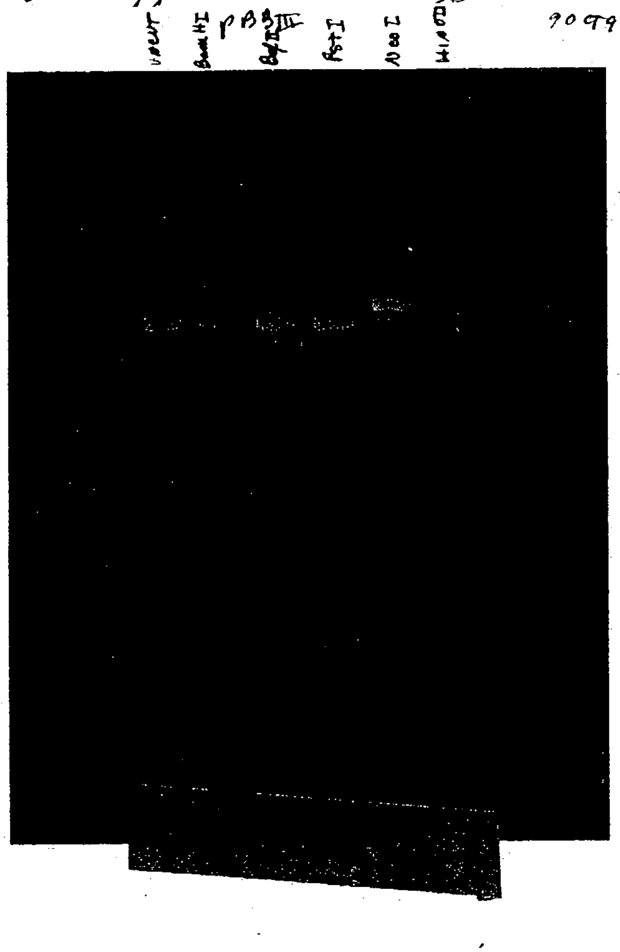
FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE IN HUMANS OR ANIMALS.

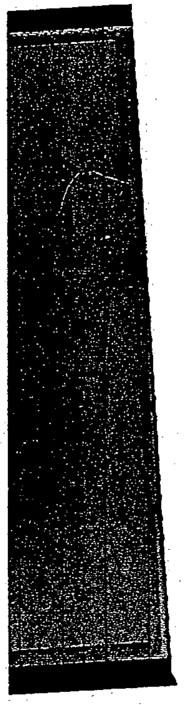
pBBIII - Maxiprep - Restriction endonuclease analysis
Set up assays w/ 1.5 ug/ul maxiprep DNA to determine if all the ~~sites~~ restriction endonuclease sites of the multiple cloning site of pBBIII are intact.

R.E.	Temp (°C)	React Buffer
Bam HI	37°C	3
Bgl II	"	3
Pst I	"	2
Nco I	"	3
Hind III	"	2

16 ul H$_2$O
2 ul React buffer
1 ul pBBIII (1.5 ug)
1 ul R.E

Inc 1H @ 37°C. Ice 10 min.
Analyze on a 1% SeaPlaque agarose gel - 90992





```
              SINGLE λ          02           Abs
                                                      Read average = 01
S#     600.0                S#    600.0               S#    600.0
   25ml preps - pBBIII - Started at 7:10A
1       0.0000 LB broth
2       0.0305 55
3       1.6882 81
4       1.3458 82
5       1.5551 83
6       0.0428 99
```

```
  λ         ABS      SOURCE      11:52     CELL
600.0     0.0488   Visible 10/15/92    1
```

```
              SINGLE λ          02           Abs
                                                      Read average = 01
S#     600.0                S#    600.0               S#    600.0
     25ml broth  pBBIII - Started @ 7:10A
7       0.0000 LB broth
8       1.6997 #55
9       1.7633 # 99
10      0.2446
11      0.2101
12      0.2104
```

GIBCO BRL LIFE TECHNOLOGIES, INC.
GAITHERSBURG, MARYLAND 20898

5219SA

Lot # BHX104

5'-GCCN NNN↓NGGC-3'

3'-CGGN↑NNN NCCG-5'

Bgl I

1,000 units; 10 U/µl
Store at +4°C

Bgl I is isolated from Bacillus globigii. One unit is the amount of enzyme required to cleave 1 µg of lambda DNA in one hour at 37°C in the appropriate buffer.

Storage Buffer:
50 mM Tris-HCl (pH 8.0)
200 mM NaCl
1 mM Na_2EDTA
7 mM 2-mercaptoethanol
1 mM NaN_3
500 µg/ml BSA
50% (v/v) glycerol

Reaction Conditions:
Use REact® 2. REact® 2 is the 10X concentrate assay buffer supplied for use with Bgl I. Dilute 1 part in 10 in final reaction mixture.
Final concentration:
50 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$
50 mM NaCl
Assay at 37°C.
Store buffer at +4°C or -20°C.

Quality Control Data:

Endonuclease Assay: Cleavage only at recognition site at 100-fold excess digest of λ DNA.

Exonuclease Assay: Using 1 pmol radiolabeled termini in a 50 µl reaction and up to 20 units of enzyme for one hour, the following results were observed:
1 unit of enzyme removed ≤0.3% label from 5' ends.
1 unit of enzyme removed ≤0.3% label from 3' ends.

Ligation/Recut Assay: On SV40 DNA: >95% ligation in 1 hr. at 22°C. 100% recut.

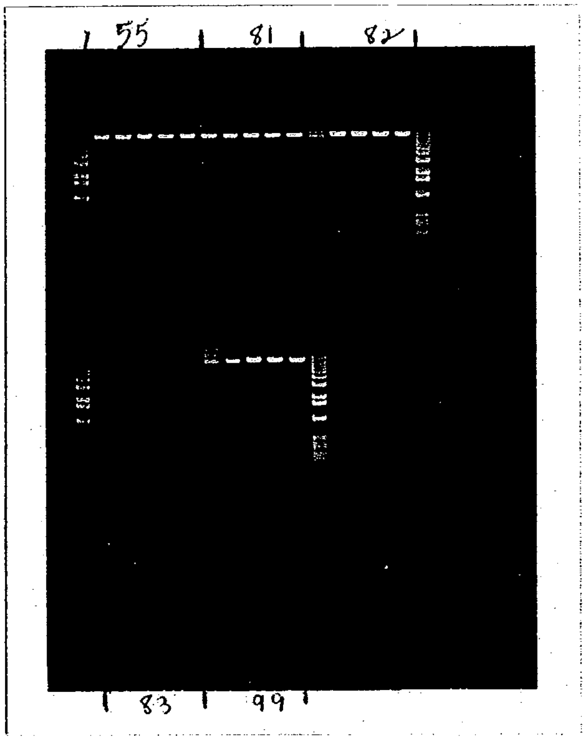
REFER TO THE BRL CATALOGUE AND REFERENCE GUIDE FOR NOTES ON CONDITIONS WHICH AFFECT ENZYME ACTIVITY.

Minipreps and Maxipreps of additional pBBIII colonies. Previous prep of #39 resulted in no enzymes cutting. 5 more colonies were selected from grid plates - #55, 81, 82, 83, 99. All were transferred from grid plate w/ toothpick and placed in 2-12ml LB/AMP broths and alle ~~colonies~~ bacteria allowed to grow O.N. @ 37°C at 2001 pm.

Minipreps of #55, 81, 82, 83, 99 were done by Cathy Schmidt (See p 35 of Notebook 3460)

Enzymes: Bam HI (React 3)
Bgl II (")
Pst I (React 2)
Nco I (React 3)
Hind III (React 2)

16 ul H_2O
2 ul React buffer
1 ul of miniprep DNA
1 ul enzyme



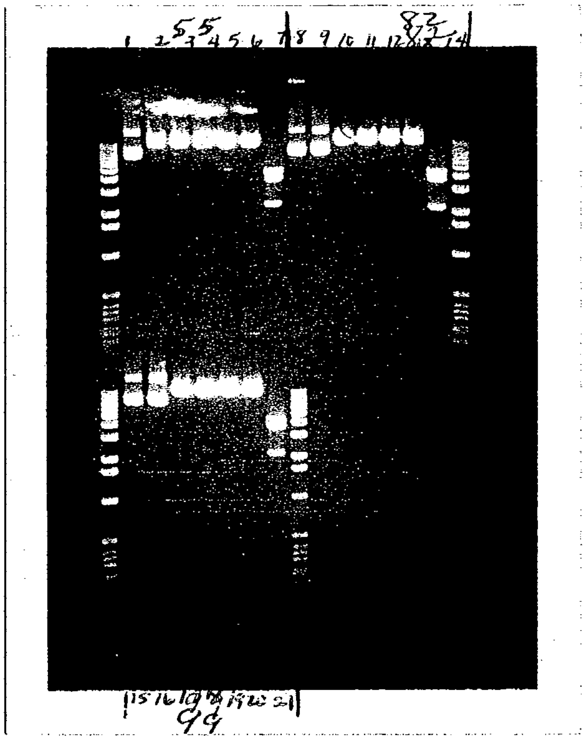
Maxipreps of #55, 82, 99 were done.
Restriction analysis with Bam HI (React 3)
Bgl II (")
Pst I (React 2)
Nco I (React 3)
Hind III (React 2)
Bgl I (React 2)

```
        DUAL λ        01          Abs      Factor= 1.000
                                           Read average = 01
S#      260.0     280.0     λA/λB    S#     260.0     280.0     λA/λB
        5ul in 500 ul H2O -     500ul total volume in sample
4       0.0009    0.0009    1.000
5       0.1585    0.0987    1.605  pBBIII #55   800.4ug/ml = 0.8ug/ul
6       0.4223    0.2370    1.781    "    #82   2,132.6ug/ml = 2.1ug/ul
7       0.1313    0.0667    1.967    "    #99   663 ug/ml = 0.663ug/ul


 λ          ABS       SOURCE     10:03      CELL
260.0      0.0012     UV/Vis    10/19/92     1
```

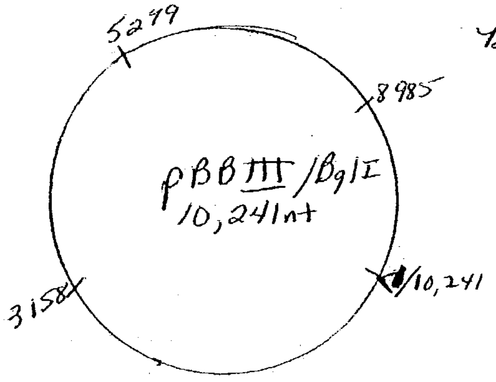
15ul H_2O
2ul DNA (#55, 99)
2ul React buffer
1ul enzyme

16ul H_2O
1ul DNA (#82)
2ul React buffer
1ul enzyme



1 - #55 - uncut
2. " - BamHI
3 " - Bgl II
4 " - PstI
5 " - Nco I
6 " - HindIII
7 " - BglI
8 #82 - Uncut
9 - BamHI
10 - ~~PstI~~ BglII
11 - ~~NcoI~~ PstI
12 - ~~HindIII~~ NcoI
13 ~~Bc~~ HindIII
14 - BglI

15 - #99 - uncut
16 - " BamHI
17 - " BglII
18 - " PstI
19 - " NcoI
20 " HindIII
21 " BglI



BglI = 3 sites: +3158, +5279, +8985

$$\begin{array}{rrrr} 10241 & 1256 & 5279 & 8985 \\ \underline{8985} & \underline{3158} & \underline{3158} & \underline{5279} \\ 1256 & \boxed{4,414} & \boxed{2,121} & \boxed{3,706} \end{array}$$

Primers for the cloning of the VEE virus structural genes into the BamHI and HindIII restriction endonuclease recognition sites of the baculovirus pBluebac III transfer vector.

VC-F1 ✓
BamHI — 31 — CAPSID — 51
5'>GGT CTA GGA TCC GCC GCC ACC ATG TTC CCG TTC CAG CCA ATG<3'
clamp — Kozak

VE3-F2 (NO A) ✓
BamHI — 856 — E3 — 872
5'>GGT CTA GGA TCC GCC GCC ACC ATG TCA CTA GTA CCA CCA T<3'
clamp — Kozak — G

VE3-F1 (A): ✓
BamHI — purine — 856 — E3
872 5'>GGT CTA GGA TCC GCC GCC ACC ATG GCT TCA CTA GTG ACC ACC AT<3'
clamp — Kozak — Alanine

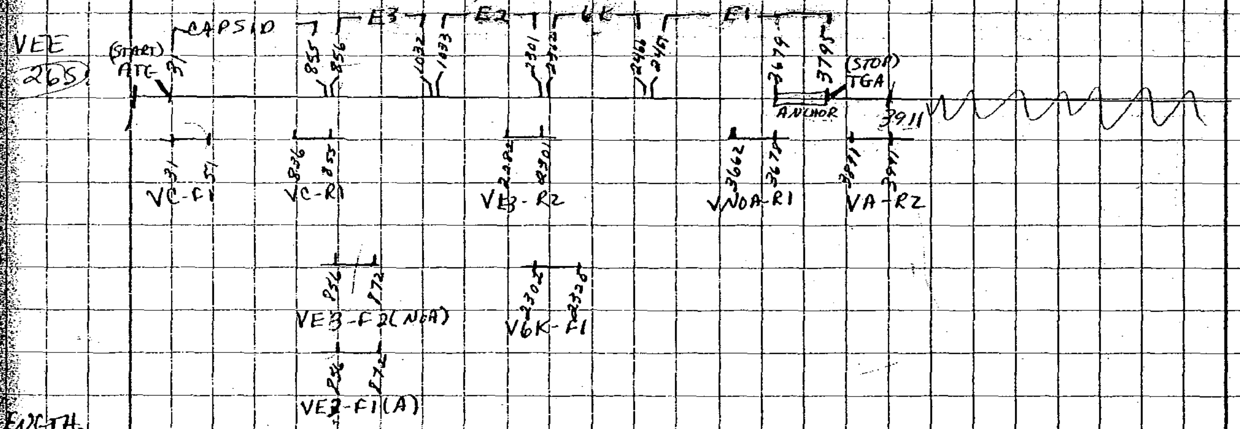
V6K-F1: ✓
BamHI — 2302 — 6K
2320 5'>GGT CTA GGA TCC GCC GCC ACC ATG GAG ACC ACC TGG GAG TCC T<3'
Clamp — Kozak

VC-R1 ✓
Bgl II — 855 — CAPSID — 836
5'>CTC GGC TAG ATC TAA TCC ATT GCT CGC AGT TCT CCG<3'
clamp — STOP

VNOA-R1 ✓
HindIII — 3678 — E1 — 3662
5'>CTC GGC TTT CGA ATC ACT ATT TTG ACA CCG CGG CTG<3'
clamp — STOP — STOP

VA-R2: ✓
HindIII — 3911 — NONE CODING 3'END — 3891
5'>CTC GGC TTT CGA AGA ATA TTA AAA ACA AAA TCC G<3'
clamp

VE3-R2: ✓
HindIII — 2301 — E2 — 2282
5'>CTC GGC TTT CGA ATC ACT AGG CCC GGG CAG TGC GGG CGC<3'
clamp — STOP — STOP



LENGTH			
861	1)	VC-F1 → CAPSID ← VC-R1	CAPSID
3,095	2) ✓	VE3-F1 → E3 | E2 | 6K | E1 ANCHOR ← VA-R2	E3(A), E2, 6K, E1
3,090	3)	VE3-F2 → E3 | E2 | 6K | E1 ← VA-R2	E3(NO A), E2, 6K, E1
1,491	4)	VE3-F1 → E3 | E2 ← VE3-R2	E3(A), E2
1,598	5)	V6K-F1 → 6K | E1 ← VA-R2	6K + E1
2,868	6)	VE3-F1 → E3 | E2 | 6K | E1 ← VNOA-R1	E3(A), E2, 6K, E1 (NO AN
3,914	7) ✓	VC-F1 → CAPSID | E3 | E2 | 6K | E1 ← VA-R2	CAPSID, E3, E2, 6K, E1 (26

```
             - 394 Synthesis Setup Listing -        (Version 1.01)

Column 1                                        11:53:11A ,10/20/92

Run ID   :
Cycle    : Cyc02 user
End Proc: End CESS      (DMT = On )
Sequence: VNOAR1

Total bases =  36
        A=  6, G=  8, C= 11, T= 11, 5=  0, 6=  0, 7=  0, 8=  0
        (mixed bases=  0)

MW:  11729.2

     5'> CTC GGC TTT CGA ATC ACT ATT TTG ACA CCG CGG CTG <3'
```

(date corrected by hand: 10/~~20~~21/92)

$Em = A(16000) + C(7000) + G(12000) + T(9600)$

$= 6(16000) + 11(7000) + 8(12000) + 11(9600)$

$= 96000 + 77{,}000 + 96{,}000 + 105{,}600$

$Em = 374{,}600$

$\text{Molarity} = \frac{OD_{260} \cdot \text{dil factor}}{Em}$

$= \frac{0.2803 \cdot 101}{374{,}600}$

$= 0.0000755$

Molarity = 75.5 pmol/ul

PCR: Need 100 pmol/ul

Use 1.3 ul/rxn

)ate: 12/02/92

0 kcal)

bases)

```
SW     260.0     280.0    λA/λB    SW     260.0     280.0    λA/λB
```

5ul in 500ul H2O = 101 dil   200 ul total sample

```
 1      0.0000    -0.0000  -0.000
 2      0.2436     0.1584   1.537  VE3R2 22Oct92
 3      0.2803     0.2061   1.359  VNOA R1  "
 4      0.3653     0.1946   1.876  VAR2 21Oct92
```

```
   λ          ABS       SOURCE      10:52     CELL
260.0      0.0007     UV/Vis   10/23/92      1
```

VC-F1
31
-21
10

10 - 871 = 861

VE3-F1
856
-27
829

3924
829
3095

VE2-R2
856
24
834

3924
834
3090

VGK-F1
2302
24
2326

392
-232
159

VC-R1
855
+16

VA-R2
3911
13

VE3-R2
2301
19

2320
829

VNOA-R1
3678
19

369
829

15mM 1.5 mM in 100 µl

15mM/µl

PERKIN ELMER CETUS
761 Main Ave., Norwalk, CT 06859

GeneAmp® PCR Reagent Kit with
AmpliTaq® DNA Polymerase (250 Units)

(Deoxynucleoside triphosphate: DNA Deoxynucleotidyltransferase, EC 2.7.7.7)

NOT FOR DIAGNOSTIC OR MEDICAL USE
RESEARCH USE ONLY
See notice to purchaser

Part No. N801-0055

The Perkin-Elmer Cetus *GeneAmp* PCR Reagent Kit is designed to perform the *GeneAmp*™ Polymerase Chain Reaction (PCR) process on all DNA templates. It provides sufficient reagents for 100 amplifications of 100 µL each. It includes full length lambda DNA and control primers for 10 control amplifications.

The *GeneAmp* PCR process is a simple and powerful method,[1,6] invented by K. Mullis and patented by Cetus Corporation, which allows *in vitro* amplification of DNA segments (including cDNA)[3] through a succession of incubation steps at different temperatures.[1,2,3,4,13] Typically, the double stranded DNA is heat denatured, the two primers complementary to the 3′ boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these three consecutive steps is referred to as a cycle. The *GeneAmp* PCR process is based on the repetition of this cycle and can amplify DNA segments by at least 10^5 fold, and potentially as high as 10^9 fold,[1] under the conditions described below.

LIST OF KIT COMPONENTS

Store the *GeneAmp* PCR Reagent Kit and *AmpliTaq* DNA Polymerase at −20°C in a constant temperature freezer. If stored under proper conditions, the product will maintain performance through the control date printed on the label.

Reagent	*Volume*	*Concentration*	*Comments*
AmpliTaq DNA Polymerase	50 µL	5 U/µL	Extends primer during PCR amplification
dATP	320 µL	10 mM	Deoxynucleosidetriphosphates dissolved in water, pH 7.0.
dCTP	320 µL	10 mM	
dGTP	320 µL	10 mM	
dTTP	320 µL	10 mM	
[10X] Reaction Buffer (See NOTE below)	1.4 mL		Provides preferred pH and ionic strength for amplification.
Control Template	100 µL	1 µg/mL	Whole bacteriophage lambda DNA as a template for PCR.
Control Primer #1	50 µL	20 µM	Primer pair defines a 500 base segment on the lambda target DNA: nucleotides 7131 through 7630 (see on page 4).
Control Primer #2	50 µL	20 µM	

NOTE: [10x] Reaction Buffer composition.
(solution has been autoclaved)

100 mM Tris-HCl, pH 8.3 (at 25°C)
500 mM KCl
15 mM $MgCl_2$
0.01% (w/v) gelatin (Sigma, Cat. No. G2500, St. Louis, MO)

AmpliTaq DNA Polymerase

The key component in the *GeneAmp* PCR Reagent Kit is *AmpliTaq* DNA Polymerase **(Part No. N801-0060)**. Perkin-Elmer Cetus' *AmpliTaq* DNA Polymerase is a recombinant, thermostable, 94kDa DNA polymerase encoded by a modified form of the *Thermus aquaticus* DNA polymerase gene which has been inserted into an *Escherichia coli* host.[8] *AmpliTaq* DNA Polymerase and native *Taq* DNA Polymerase are covered by U.S. Patent 4,889,818 issued to Cetus Corporation, and licensed to Perkin-Elmer Cetus Instruments. Additional patent applications on genes encoding thermostable DNA polymerases, and methods and formulations for stable preparations of thermostable DNA polymerase are pending. Its features are described on page 4.

NOTICE TO PURCHASER ABOUT LIMITED LICENSE

This product is designed for use in the *GeneAmp* Polymerase Chain Reaction (PCR) process covered by patents issued to Cetus Corporation. The seller has a limited license under such patent rights, and purchase of this product includes a fully paid-up, limited, non-exclusive sublicense under such patent rights to use this product to perform the *GeneAmp* PCR process only for research and development activities and for industrial quality assurance testing. For this purpose the license for "research and development" includes the development of commercial products, such as new therapeutic products. No license is granted for any other uses, for example (1) the diagnosis or detection of disease or other health-related condition in humans, animals or plants, or (2) genetic analysis for identification of individuals for forensic or other purposes. No other license is granted expressly, impliedly, or by estoppel. For information concerning the availability of additional licenses to practice the PCR process, contact Cetus Corporation, Director of Business Development, PCR Division, at 1400 Fifty-Third Street, Emeryville, CA 94608, Tel. (415) 420-3300.

PCR - 23 Oct 92

DNA	λC	ZOWAT #11						
PCR PRIMERS (100 pmol/rxn)	Pr#1	✓ VC-F1	✓ VE3-F1	✓ VE3-F2	✓ VE3-F1	✓ V6K-F1	✓ VE3-F1	VC-F1
	Pr#2	VC-R1	VA-R2	VA-R2	VE3-R2	VA-R2	VNoA-R1	VA-R2
		①	②	③	④	⑤	⑥	⑦
Expected length (bp)	500	861	3095	3090	1491	1585	2868	3914
H_2O (ul)	70.5	79.1	78	78.3	77.4	78.8	77.8	78.7
Template (ul) (Miniprep)	1	0.5 →						
Pr#1 (ul) or FORWARD PR.	5	1.2	1.9	1.6	1.9	1.1	1.9	1.2
Pr#2 (ul) or REVERSE PR.	5	0.7	1.1	1.1	1.7	1.1	1.3	1.1
10X RXN BUFFER 10mM dNTP mix		MM = 18 ul →						
Taq pol (ul)	0.5	→						
		20.9	22	21.7	22.6	21.2	22.2	24.3
TV	100	100	100	100	100	100	100	100

Vortex, microfuge

Mineral oil (ul)	100 →	

Thermocycler Program - User 404 File 40

Step cycle: 30 sec @ 95°C } 35 cycles
35 sec @ 52°C
3 min @ 72°C

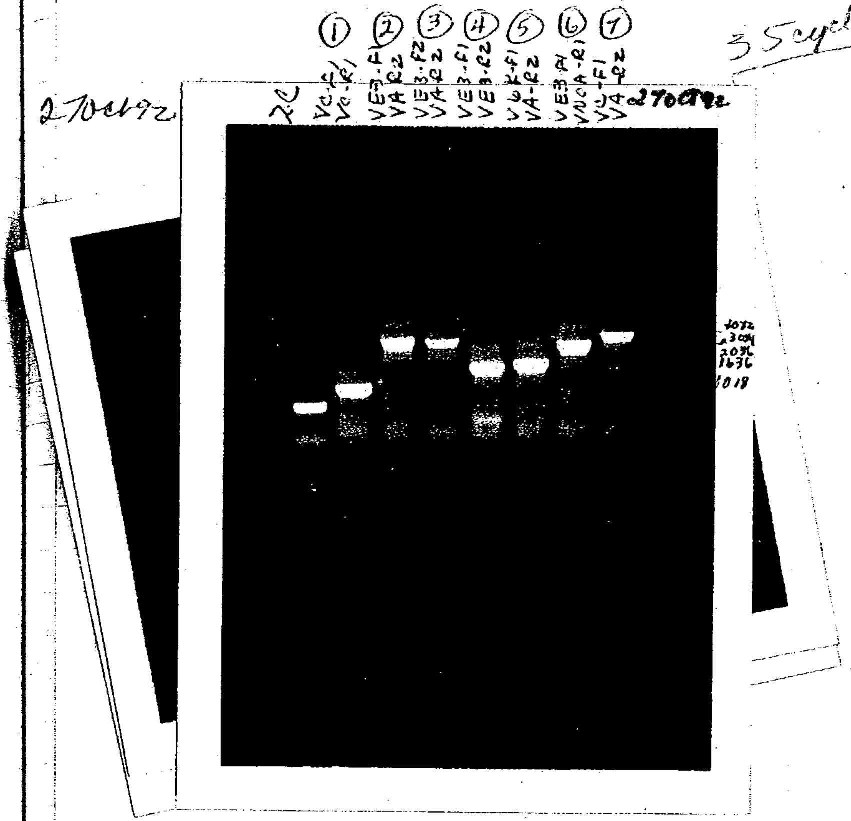
Delay cycle: 10 min @ 72°C (File 99)

Soak cycle: 4°C File 99

MM:
dATP 9 x 2 = 18 ul
dCTP 9 x 2 = 18 ul
dGTP 9 x 2 = 18 ul
dTTP 9 x 2 = 18 ul
10x Rxn buffer 10 x 9 = 90 ul
162 ul
18 ul/rxn

27Oct92

3 5 cycles



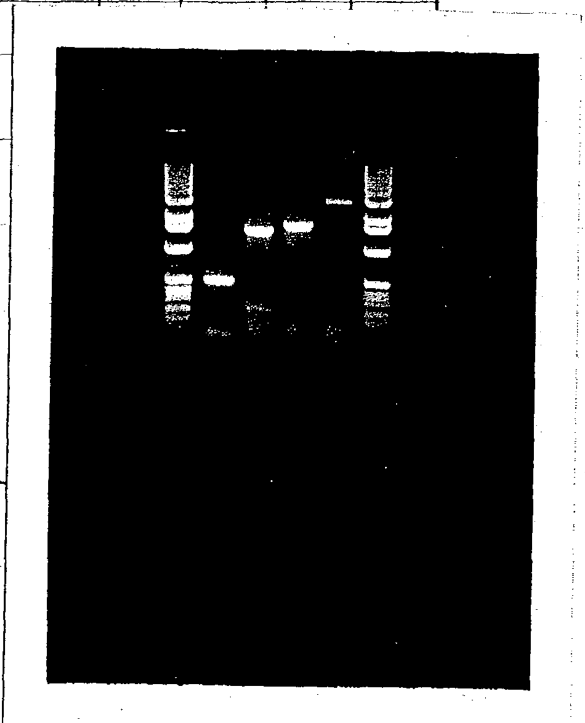
Expected band lengths

1 - 861 = CAPSID
2 - 3,095 = E3(A), E2,6K
3 - 3,090 = E3(NcA), E2,6
4 - 1,491 = E3(A), E2
5 - 1,598 = 6K + E2
6 - 2,868 = E3(A), E2,6K,E
7 - 3,914 = 26S

No MgCl2 optimization was done.
5X amplifications of #2, #3, #7 were done.

PCR - 5X amplifications
PCR - 27 OCT 92

DNA	λC	ZQWATH#11 #2	#3	#7
PCR Primers	Pr#1	VE3-F1	VE3-F2	VC-F1
(100 pmol/μm	Pr#2	VA-R2	VA-R2	VA-R2
Expected length (bp)	500	3,095	3,090	3,914
H_2O (ul)	70.5	390	391.5	393.5
Template (ul) (minipep)	1	2.5	2.5	2.5
Pr#1 (ul) or FORWARD PR.	5	9.5	8	6
Pr#2 (ul) or REVERSE PR.	5	5.5	5.5	5.5
Master mix (ul)	18	90	90	90
Taq pol (ul)	0.5	2.5	2.5	2.5
TV	100	500	500	500



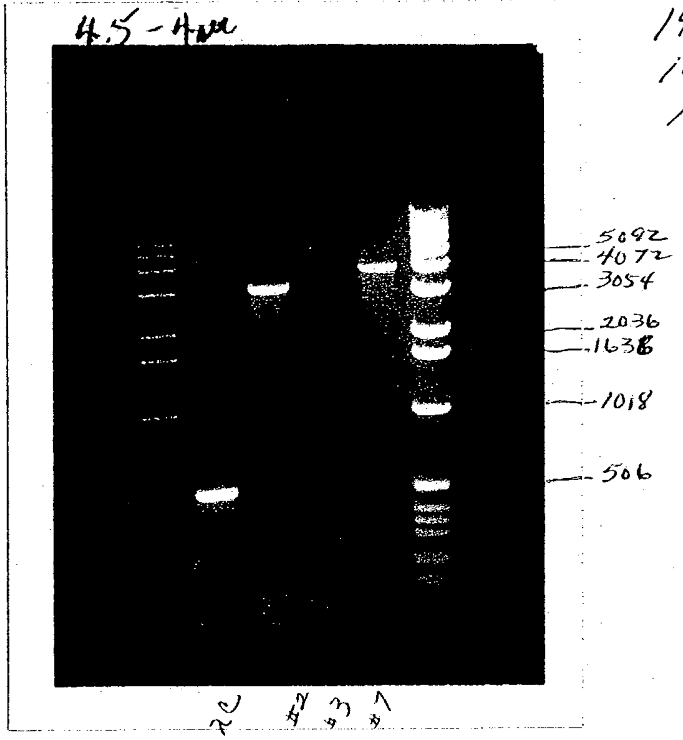
Vortex, microfuge

Mineral oil (ul) 100 →

Thermocycler Program: User 404, File 40
Stepcycle: 30 sec @ 95°C
30 sec @ 52°C
3 min @ 72°C } 35 cycles
Delay cycle: 10 min @ 72°C (File 42)
Soak cycle: 4° C (File 99)

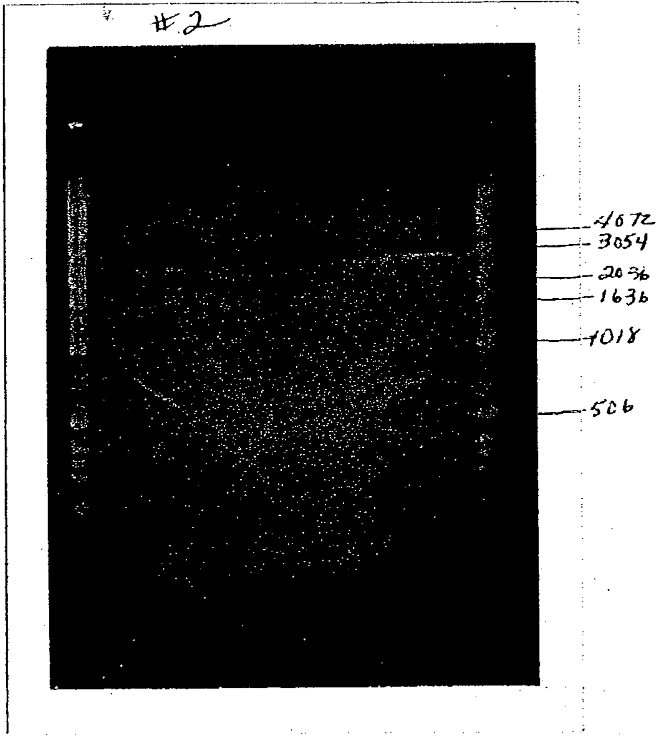
M M: dATP: 18 x 2 = 36
dCTP: 18 x 2 = 36
dGTP: 18 x 2 = 36
dTTP: 18 x 2 = 36
10X RXN buffer: 10 x 18 = 180

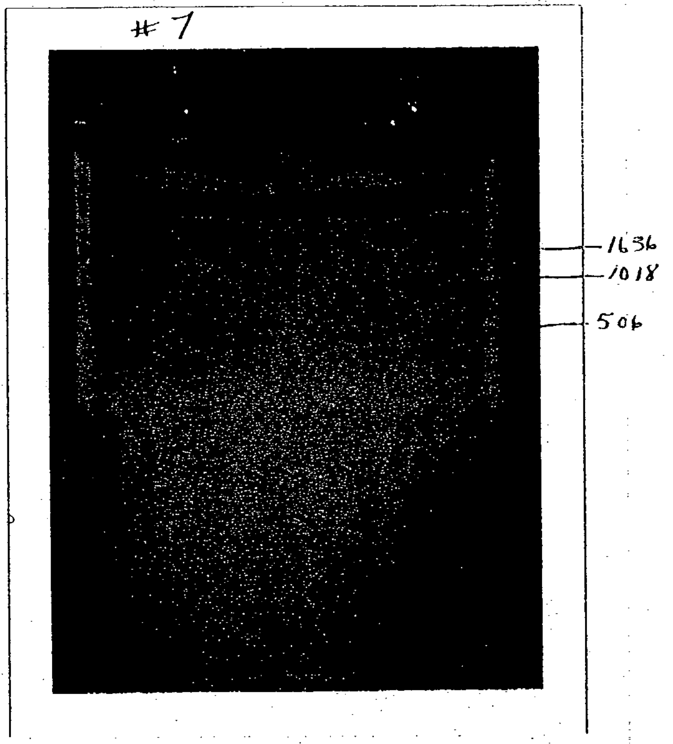
30 cycles



1% Seakem LE agarose
100V
10uL of 5X amplification reaction
Expected lengths (bp)
# 2 - 3095 = E3(A), E2, 6K, E1
# 3 3090 = E3(NoA), E2, 6K, E1
# 7 3,914 = 26S

Remaining sample was added to 1% Seaplaque (low melting point agarose) along with 1Kb DNA ladder in outside lanes. No EtBr was added to gels or buffer.





Bands were excised from gel after 10 min staining w/ EtBr using LONG wavelength UV. The gel slice was divided into 8 sections and placed in 1.5 ml Eppendorf tubes.

~~The~~

PCR Prep DNA purification

Used Promega Magic PCR Preps DNA Purification System for Rapid Purification of DNA Fragments. Cat# A7170.

1. Separate the PCR reaction products by electrophoresis in a low gelling/melting temperature agarose.
2. Excise the desired DNA band using a clean sterile razor blade or scapel. The band should be visualized with a medium or long wavelength (eg. > ~~3000~~ 300 nm) UV light + should be excised quickly to minimize exposure of the DNA to UV light. The band should be isolated in ≤ 300 mg of agarose (~ 300 μl)
3. Transfer the agarose slice to a 1.5 ml microcentrifuge tube and incubate the sample @ 70°C until the agarose is completely melted.
4. Add 1 ml of Magic PCR Preps Resin to the melted agarose slice. Vortex for 20 sec. to mix.

PCR Product Purification Using a Vacuum Manifold:

Promega Vac-Man Laboratory Vacuum Manifold (Cat # A7231)

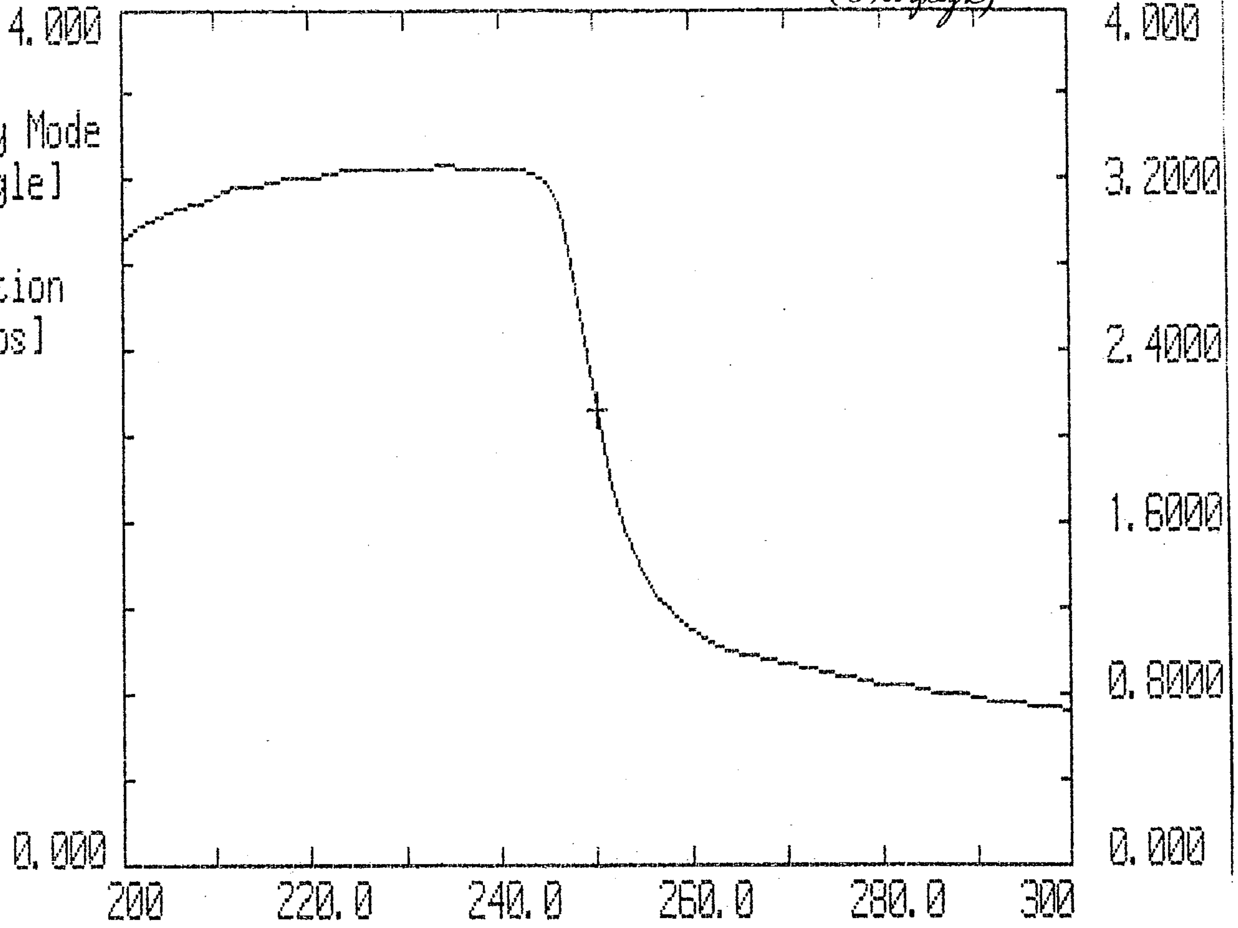
Also needed: 80% isopropanol (2-propanol, reagent grade)
d.H_2O or TE buffer
3 ml luer-lock ~~[illegible]~~ syringe

1. For each PCR product, prepare one Magic Minicolumn. Remove the plunger from a 3 ml disposable syringe and set aside.
2. Attach the syringe barrel to the luer-lock extension of each Minicolumn. Insert the tip of the Minicolumn/syringe barrel assembly into the vacuum manifold.
3. Ⓟipet the resin/DNA mix into the syringe barrel. Apply a vacuum (H_2O aspirator) to draw the resin/DNA mix into the Minicolumn.
4. To wash the column, add 2 ml of 80% isopropanol to the syringe barrel, and apply a vacuum to draw the solution through the Minicolumn.
5. Dry the resin by continuing to draw a vacuum for an additional 1-2 minutes. Remove the syringe barrel and transfer the Minicolumn to a 1.5 ml microcentrifuge tube. Centrifuge the Minicolumn for 20 sec @ 12,000 x g to remove any residual isopropanol.
6. Transfer the Minicolumn to a new microcentrifuge tube. Apply 50 μl of water or TE buffer to the minicolumn + wait 1 min (the DNA will remain intact on the minicolumn.

Scan # 01.01 #2 - DNA PREP after Minicolumn (Promega)

Display Mode
[Single]

Function
[Abs]

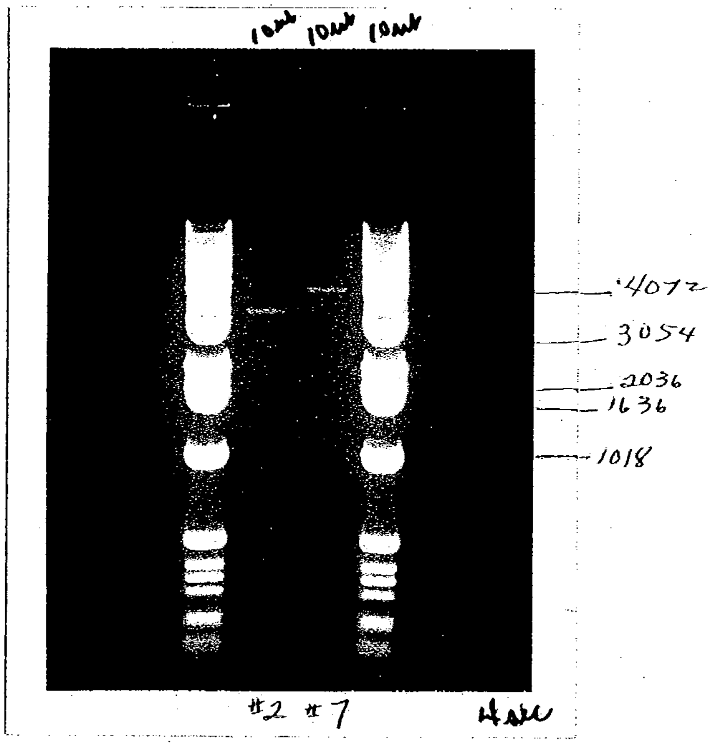


2400 nm/min Smoothing [no] pts.

TRACE

λ	ABS	SOURCE	10:02	CELL	λ	ABS
300.0	-0.3124	UV/Vis	10/29/92	1	250.00	2.1274

for up to 30 min.). Centrifuge the Minicolumn for 20 sec @ 12,000 x g to elute the bound DNA fragment.
7. Remove and discard the Minicolumn. The purified DNA may be stored in the microcentrifuge tube @ 4°C or -20°C.



#2 = VEEGP's ≅ 3095bp
#7 = VEE 26S = 3,914 bp

```
         DUAL λ        01          Abs       Factor= 1.000
                                             Read average = 01
S#     260.0     280.0     λA/λB     S#     260.0     280.0     λA/λB
```

50ul sample   Total volume = 400ul

```
1      1.1180    0.8487    1.317 #2
2      1.0286    0.7833    1.313 #7
```

#2 1.118 x 50 = 55.9 ug/ml = 22.36 ug total sample
= 0.0559 ug/ul

#7 1.0286 x 50 = 51.43 ug/ml = 0.05143 ug/ul
20.6 ug total sample

```
  λ        ABS       SOURCE      09:55     CELL
260.0    1.0314     UV/Vis    10/29/92    sgle
```

Digestion of VEE GP (3095 bp) and VEE 26S (3,914 bp) PCR products with Bam HI and Hind III and ligation of Digested Product.

Have the following PCR product DNA:

VEE GP (3,095) = Prep of 29 Oct 92 - MC (minicolumn prep) - QU
3,095 bp ~~0.0514~~ 0.0559 ug/ul

VEE 26S (3,914) = Prep of 29 Oct 92 - MC - QU, 3,914 bp
0.05143 ug/ul

Restriction Endonuclease Analysis

1. All reactions are assembled on ice in 1.5 ml microcentrifuge tube.
2. Use 0.5-1.0 ug of ~~plas~~ DNA per reaction. Set up ~~3. Include in each reaction a~~ 4 tubes for each sample.
3. Standard Reaction
   - 2 ul 10X buffer
   - 0.5-1.0 ug DNA
   - 1-2 ul restriction enzyme: 2-10 U
   - H_2O to 20 ul
4. Specific reaction

VEE GP	VEE 26S
17 ul Prep of 29 Oct 92 (0.95 ug)	17 ul Prep of 29 Oct 92 (0.87 ug)
2 ul React 2 buffer	2 ul React 2 buffer
0.5 ul Hind III	0.5 ul Hind III

30 Oct 92/ 11:18A Incubate 2H @ 37°C
Add 0.1 ul 5M NaCl, 0.5 ul BamHI

30 Oct 92/ 1:00P Incubate 2H @ 37°C. Ice for 10 min.

5. Extract 2X with equal volume of TE saturated phenol: chloroform: isoamyl alcohol (25:24:1). Vortex 1 min. Microfuge 12,000 x g for 2 min. Extract 2X w/ equal volume of chloroform. 30 Oct 92/ 2:40P

6. Precipitate w/ 1/10 volume of 2M NaOAc, pH 4.5 and 2 volumes 100% EtOH.

7. Freeze on dry ice for 10 min. Microfuge for 15 min @ 4°C.

8. Wash the pellet w/ 500 ul cold 70% EtOH. Dry ice for 5 min. Microfuge for 5 min @ 4°C.

2 Nov 92 9. Resuspend in ~~50~~ 100 ul H_2O. ~~Quantitate $A_{260/280}$~~.

10. Pre-rinse 6 - Centricon 100s. Insert sample reservoir into filtrate cup. Pre-rinse to get rid of the azide by centrifuging 2 ml of deionized H_2O through the device. Spin device in the concentration mode (2700 rpm in a SS-34 rotor) until at least half the rinse passes into the filtrate cup (~5 min). Invert device and spin @ 2500 rpm for 2 more min to remove the remaining rinse. Add PCR/R.E cut DNA

```
          DUAL  λ          01            Abs       Factor= 1.000
                                                   Read  average = 01
S#       260.0       280.0      λA/λB      S#     260.0      280.0      λA/λB

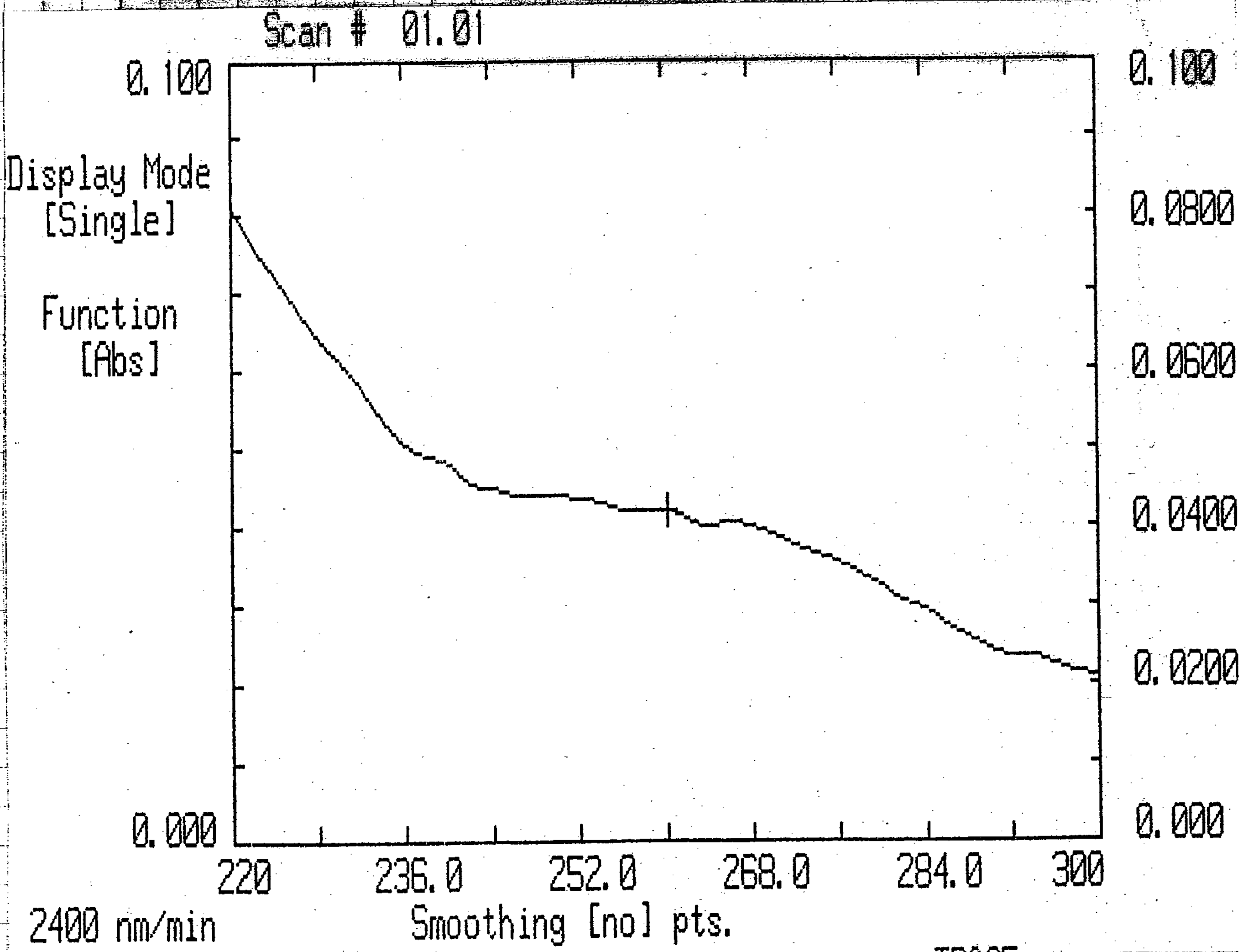
1        0.0016      0.0011     1.375
2        0.0516      0.0396     1.302
3        0.0628      0.0494     1.270
```

50 µl sample

#2 x50µg/ml = 2.58µg/ml = 0.00258µg/µl = 0.129µg

#1 x50µg/ml = 3.14µg/ml = 0.00314µg/µl = 0.157µg

```
 λ          ABS       SOURCE       15:17       CELL
50.0       0.0642     UV/Vis     11/02/92      sgle
```



```
                                                        TRACE
 λ          ABS       SOURCE      15:22      CELL          λ          ABS
200.0      0.3122     UV/Vis    11/02/92      1         258.00     0.0421
```

to sample reservoir. Centrifuge the Centricon 100 at 2700 rpm for 30 min. Dilute to 2 ml with deionized H_2O and centrifuge again. Add 500 ul H_2O. Centrifuge 20 min. Add retentate cup, ~~inv~~ invert unit and centrifuge at 2500 rpm for 2 min to transfer concentrate into cup. Analyze @ $A_{260/280}$. 1.00 unit = 50 ug/ml. (See p92

11. Speed vac to dryness. Add 7 ul H_2O to each tube.

12. To determine if R.E. cut the PCR product the BamHI/Hind III treated PCR products were incubated with T4 DNA ligase and analyzed for presence of multimers by gel electrophoresis. An uncleaved PCR product will lack 5' phosphate and will not self-ligate (Biotechniques 9:3 (1990) 305, 306.

ov92 13. Set up the following ligations
~~2 ul H_2O~~ 2 ul 5X ligase buffer
1 ul PCR product / BamHI / Hind III cut
1 ul T4 DNA ligase
Ligate O N @ 16C. Heat 10 min @ 68°C. (accual = 36 min)

ov92 14. ~~The~~ One of the reactions was then recut.
1 of ligated tubes were cut w/ BamHI and 1 cut with Hind III.

7 ul H_2O	7 ul H_2O
10 ul of ligated DNA	10 ul ligated DNA
2 ul of React 3 buffer	2 ul React 2 buffer
1 ul BamHI	1 ul Hind III

Incubate 1 H @ 37°C. Ice 10 min. Analyze all products on 1% LE agarose gel.
Results: Not enough DNA so see anything in ligated samples.

Digestion of pBBIII transfer vector

Have maxiprep of #55, pBBIII prep of 19 Oct 92 (see p 12 of this notebook).
pBBIII = 0.8 ug/ul. Total volume sample = ~500 ul = 400 ug.
~~Add 20 ug to 1~~. Aliquot 20 ug/tube = 25 ul/tube.
Speed vac to dryness. Analyze @ $A_{260/280}$. 1.00 = 50 ug/ml.

```
        DUAL λ          01           Abs       Factor= 1.000
                                               Read average = 01
S#      260.0       280.0     λA/λB      S#      260.0       280.0     λA/λB
                             50ul sample -
1       0.0001      0.0004    .3541
2       3.0481      2.9544    1.031 pBBIII
```

```
        DUAL λ          01             Abs        Factor= 1.000
                                                  Read average = 01
S#      260.0       280.0      λA/λB      S#      260.0       280.0      λA/λB
```

5ul in 500ul H_2O = 1:101 dil

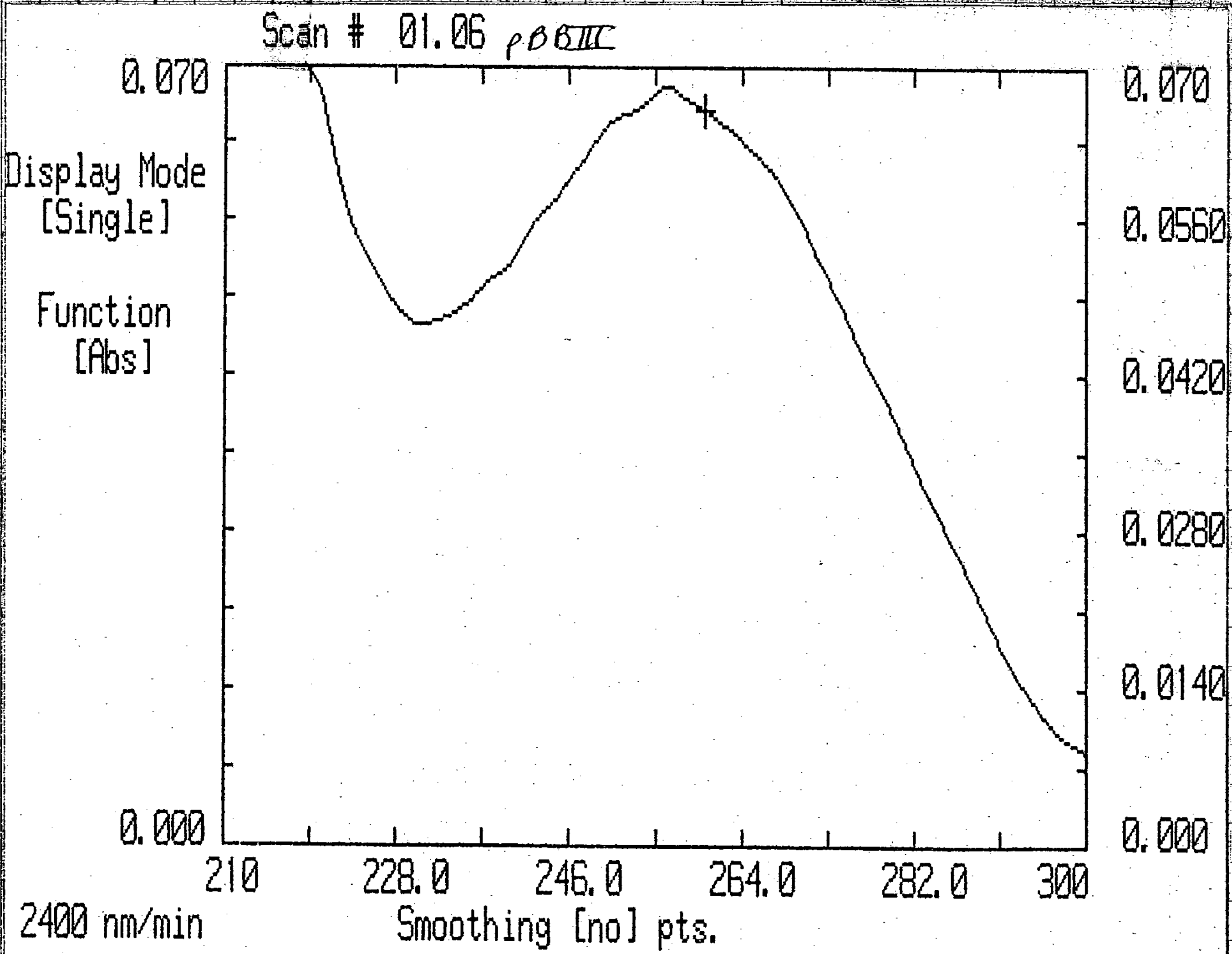
```
1      -0.0004     -0.0006     .7500
2       0.0610      0.0356     1.714
```

pBBIII

0,061 x 50 ug/ml = 3.05 ug/ml x 101 = 308.05 ug/ml = 0.31 ug/ul

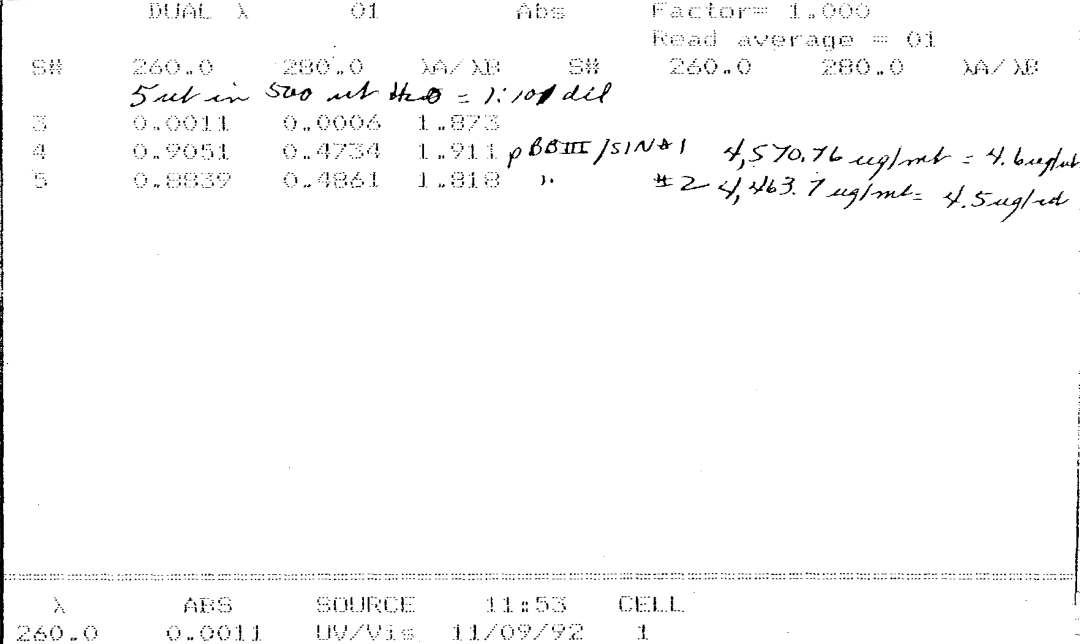
Have 50ul = 15.4 ug/sample

```
 λ        ABS        SOURCE      12:30      CELL
260.0   -0.0004      UV/Vis     11/03/92     1
```



```
                                                    TRACE
 λ        ABS       SOURCE     12:36      CELL          λ        ABS
300.0   -0.0005     UV/Vis    11/03/92     1          260.00    0.0661
```

Nov92 Have 2 tubes labeled pBBTIII/SIN 8Oct92



```
          DUAL λ         01              Abs      Factor= 1.000
                                                  Read average = 01
S#      260.0      280.0     λA/λB      S#      260.0      280.0     λA/λB
        5ul in 500 ul H2O = 1:101 dil
3       0.0011     0.0006    1.873
4       0.9051     0.4734    1.911 pBBIII/SIN#1   4,570.76 ug/ml = 4.6ug/ul
5       0.8839     0.4861    1.818  ''        #2  4,463.7 ug/ml = 4.5ug/ul

 λ         ABS       SOURCE      11:53     CELL
260.0     0.0011     UV/Vis    11/09/92     1
```

Nov92 Have VEE26S#2 & VEECP#7 preps of 29Oct92 ~~that~~ have been gel banded and DNA isolated from LMP agarose using Promega minicolumn procedure. Have ~ 350ul left each prep. Need about 10ug to do transfection of ~~[illegible]~~ E.coli.

Speed vac 180ul of #2

Speed vac 190ul of #7.

Digestion:

10X React 2 buffer	10ul
Hind III (10U/ul)	2.5ul
H_2O	

Incubate @ 37°C for 1H. Then add 2.5ul of BamHI + 1ul of 5M NaCl. ~~Heat to 5°C~~ fo ~~Quench on~~ Quench on ice for 5 min. Extract 1X w/ ϕ/$CHCl_3$. Back extract w/ 50ul 50mM Tris-HCl pH8.0. Extract again w/ ϕ/$CHCl_3$. Extract 2X w/ $CHCl_3$. Precipitate the DNA w/ 1/10 volume 3M NaOAc, pH5.2, and 2 volumes 100% EtOH. Incubate -20°C, O.N.

Nov92 Microfuge the DNA for 30min @ 4°C. Wash the pellet w/ 100 ul of 70% EtOH. Freeze @ -70°C for 5 min. Microfuge for 5 min @ 4°C.

Nov92 Quantitate spectrophotometrically @ $A_{260/280}$ - See next page - #96 - bottom of page.

from p 64 of notebook 3426

Maxiprep

V-1-1	4.2608 ug/ul	0.7ul = 3ug
V-1-2	1.9589 ug/ul	1.53ul = 3ug
C-29-2	-1.5239 ug/ul	1.97ul = 3ug
~~C-29-1~~	~~1.8861 ug/ul~~	~~1.6 ul = 3ug~~ OK
SIN-#1	4.6 ug/ul	$0.\overset{0.7}{65}$ul = 3ug
SIN #2	4.5ug/ul	0.7 ul = 3ug
pBBIII DNA	<0.31ug/ul	~~0.31~~ 9.6 ul = 3ug
~~No DNA~~ WT linear DNA only		

maxipreps of 29 Jul 92

Cationic Liposome Mediated
Transfection of SF9 cells w/ pBBIII / SIN GP construct

V92 1. Seed 2×10^6 Sf9 cells in complete TNM-FH in a 60 mm plate. Allow the cells to attach for at least 30 min at R.T.
Will have 3 plates: 2 samples of pBBIII / SINGP
and a negative control plate -
pBBIII DNA w/o SIN-GP insert
~~Need 6×10^6 Sf9 cells. Have~~
Will also repeat CCHF-HIS-NC & VEE-HIS-NC transfections of 4 Aug 92 as are having trouble plaque purifying these recombinant baculoviruses.
Will have a total of 8 samples.
Need 16×10^6 cells in 16 ml media (2 ml / 60 mm well)
Have 2.7×10^6 cells / ml
6 ml of cells + 10 ml media (Complete TNM-FH)
Added 2 ml of cells to each of 8 - 60 mm plates.

2. For each 60 mm plate to be transfected set up the following mix: Pipette 1 ml of Grace's media containing no supplements into a 1.5 ml sterile eppendorf. Add 1 ug of linear AcMNPV DNA (Invitrogen Cat # B825-03) and 3 ug of plasmid DNA (the transfer vector containing the gene of interest). Thoroughly mix the Cationic Solution then add 30 ul ~~of the~~ to the DNA / Grace's media solution. Mix thoroughly.

3. Incubate @ RT for 15 min. Remove the media from the plates and replace w/ 2 ml of Grace's media without supplements. Allow the cells to sit until the the transfection solution is within 2 min of completion. This should be approximately 10 min.

4. Remove all of the Grace's media from the cells and add the 1 ml transfection mix dropwise to the 60 mm plate.
OV92 Incubate plates on a slowly rocking platform at RT for
25A 4 H. use setting of 2.5 on a Bellco side / side rocking platform. Place platform in hood.

5. Following this incubation period add an additional 1 ml of complete TNM-FH (10% FBS, Fungizone, Gentamicin) to each 60 mm plate and incubate at 27°C in a humidified environment for 48 H.

OV92 6. 48 H later, harvest the media from the transfected plates and store @ +4°C until required. This media is the stock virus which will be used in the identification of recombinant virus by plaque assay. Replace the media with 3 ml of fresh complete TNM-FH & incubate the cells @ 27°C for a further 48 H. This is done because the cells may not yet show signs of infection even though extracellular viral particles are now being released into the

```
        DUAL λ        01          Abs       Factor= 1.000
                                            Read average = 01
S#    260.0     280.0     λA/λB      S#     260.0     280.0     λA/λB
 5ul in 500ul H2O 1:101 dil ~300ul total sample
1     0.0012    0.0013    .9162
2     0.0093    0.0049    1.908 ZOWAT#11 VE3F1/VE3R2 = 1,491 bp
3     0.0093    0.0066    1.413   "      V6KF1/VAR2   = 1,598 bp
4     0.0050    0.0013    3.888   "      VE3F1/NNoAR1 = 2,868 bp
```

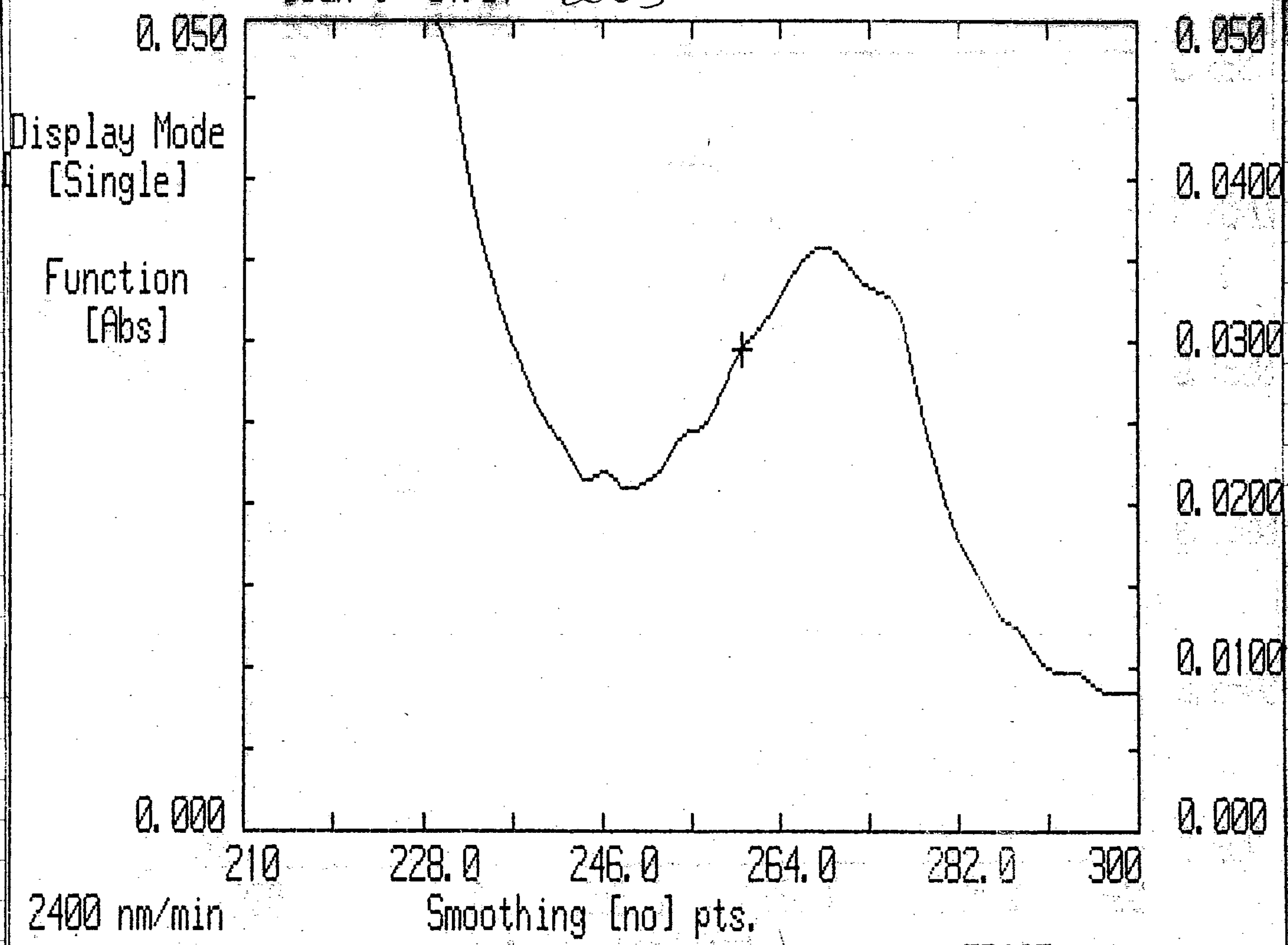
#2 46.965 ug/ml = 32ug in sample
= 0.047 ug/ul

#3 = 0.047 ug/ul

#4 = 25.25 ug/ml = ~ 7.6 ug in sample
= 0.02525 ug/ul

```
λ          ABS        SOURCE      16:09      CELL
260.0      0.0030     UV/Vis    11/13/92      1
```

Scan # 01.01 26S



```
                                                    TRACE
  λ        ABS       SOURCE     12:45     CELL          λ        ABS
300.0    -0.3261     UV/Vis   11/13/92     1         260.00    0.0295
```

media.

1. Check the cells on day ~~4~~ 6 post-transfection to visually confirm the success of the transfection. This is done using an inverted phase microscope @ 250-400x magnification. 10-50% of the cells should contain viral occlusions, which appear as refractive crystals in the nucleus of the insect cell. Other positive signs of virus infection include a 25-50% increase in the diameter of the cells, a marked increase in the size of the cell nuclei relative to the total cell volume (the nuclei may appear to "fill" the cells) and cell lysis and debris.

Plaque Purification of Recombinant Virus

1. Add 1.25 g of baculovirus agarose / 50 ml of Millipore H_2O in a 100 ml bottle. Autoclave for 15 min. 5 ml of this solution will be used per 100 mm plate.

2. Prepare 50 ml of complete TNM-FH media (10% FBS, 25 ug/ml Fungizone, 50 ug/ml Gentamicin). Place at 50°C until needed.

3. Prepare 10-fold dilutions of virus inoculum (media harvested from transfections) in the range of the expected titer. Allow 1 ml of diluted virus for each plate.
Use 10^{-2} & 10^{-3} dilutions. Make enough of each dilution to set up LAB-TEK slides also - 100 ul / well. ∴
Make up total of 2 ml of each inoculum.
10^{-2} = 20 ul of VIRUS STOCK + 1800 ul TNM-FH (complete)
10^{-3} = 150 ul of 10^{-2} dil + 1350 ul TNM-FH (complete)
It is essential that the viral inoculum is vortexed vigorously prior to the preparation of the dilutions.

4. Seed Sf9 cells at a density of 5×10^6 cells / 100 mm plate in complete media.
Have 8 transfections w/ 2 dilutions = 16 plates:
~~80 x 10^6 cells in~~ Make up enough cells for 17 plates:
85×10^6 cells in 85 ml of media. Add 5 ml of this to each 100 mm plate.

2NOV92 Have 2.8×10^6 cells / ml = 30.5 ml of cells + 54.5 ml media
LAB-TEK: 400 ul / well
Need 0.5×10^6 cells / 8 well LABTEK
4 slides total
2×10^6 cells total 12.8 ml Have 2.8×10^6 cells / ml
700 ul of cells + 12.1 ul media.

16 NOV92 Transfection plates - original.
7:30A V-1-1 - Polyhedrin observed - Cells starting to float
V-1-2 " "
C-29-2 " "

ASA 200

Set large knob on small square 200 - 24

Left Small gray knob to [3]

Right small gray knob to [X1]

Exposure#

#17 120 - took long time - aborted

30 -

p89 #2 0.0559ug/ul 10ul

#7 0.05743ug/ul 10ul.

pBBIII / HindIII / BamHI / CIAP 10ul

0.0577ug/ul

Need to cut the "Inserts" + Cent100

	Polyhedra observed	Cells starting to float.
C 29-1		
SIN #1	"	"
SIN #2	"	"
pBB III	"	"
WT DNA ONLY - NO PLASMID DNA	"	"

16NOV92 Transferred media from all of the original transformation plates to 15 ml centrifuge tubes. Centrifuged 1500 rpm for 5 min. Transferred sup to clean 15 ml centrifuge. Store 4°C.

16NOV92 LAB TEKS

		PH	PA
V-1-1	10^{-2}	+	–
	10^{-3}	(–)	–
V-1-2	10^{-2}	+	–
	10^{-3}	+	–
C-29-2	10^{-2}	+	Rare +
	10^{-3}	(–)	–
C-29-1	10^{-2}	+	Rare +
	10^{-3}	+	–
SIN#1 ~~pBBIII~~	10^{-2}	+	+
	10^{-3}	+	–
SIN#2	10^{-2}	+	+
	10^{-3}	+	–
pBBIII	10^{-2}	+	–
	10^{-3}	+	–
WT ONLY	10^{-2}	+	–
	10^{3}	+	–

16NOV92 Have following PCR preps;

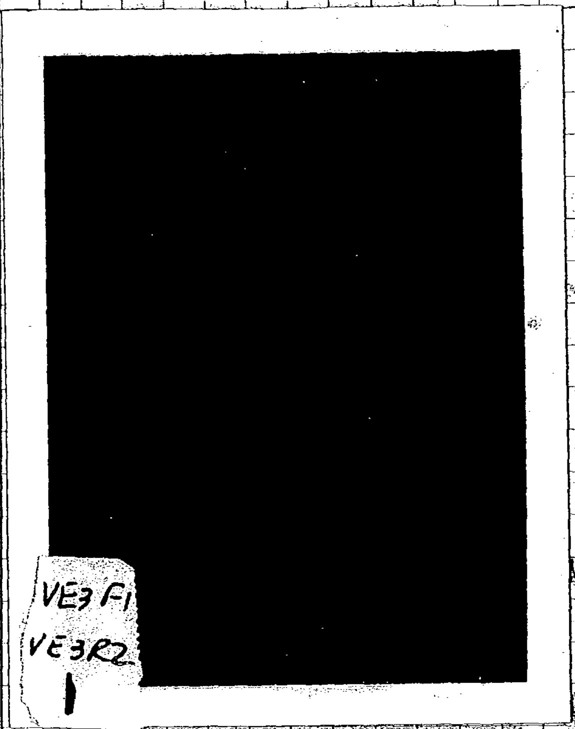
PCR DNA PREP 20WAT #11 VE3F1/VE3R2 12NOV92 } See quantitation OD260/280 p. 98 this notebook
" " V6KF1/VAR2 13NOV92
" " VE3 F/VNoA R1 13NOV92
" VEE 26S #1 29OCT92 } See quantitation OD260/280 p 89 this notebook
" VEE GP #2 29OCT92

SPEED VACCED TO DRYNESS

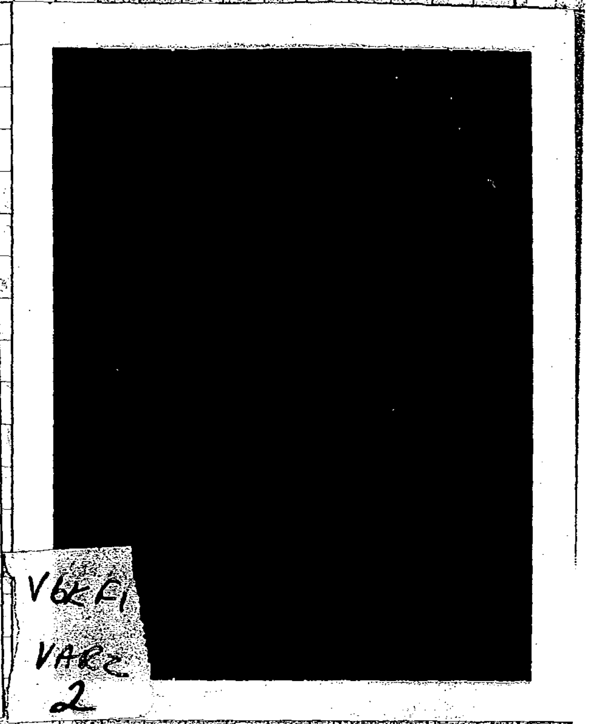
16NOV92 TOP 10 E.coli from stab to LB plate w/o AMP. Inc ON @ 37°C in walk in incubator

17Nov92 Transferred 2 colonies to each of two tubes w/ SOB media. Inc. ON w/ shaking at 200 rpm @ 37°C

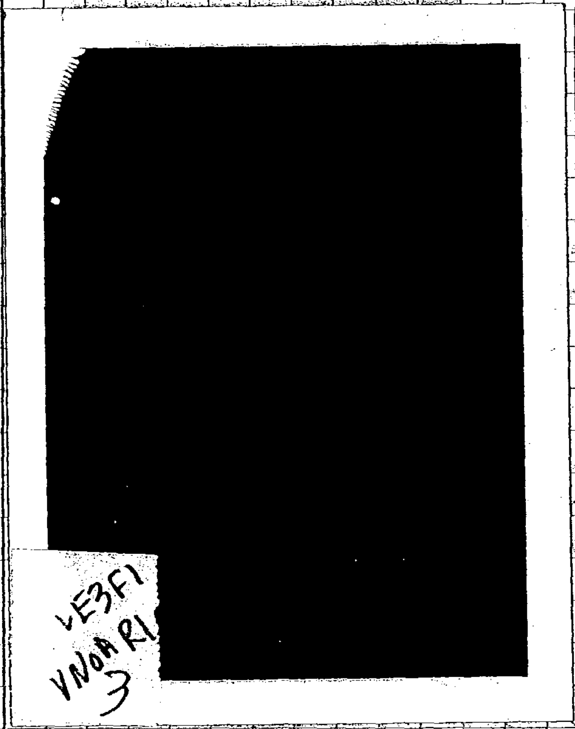
17Nov92



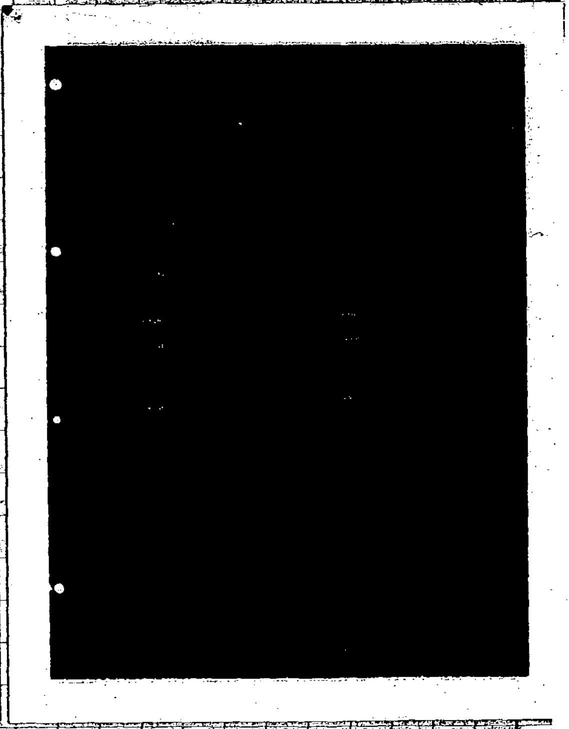
VE3 F1
VE3R2
1



V6K F1
VAR2
2

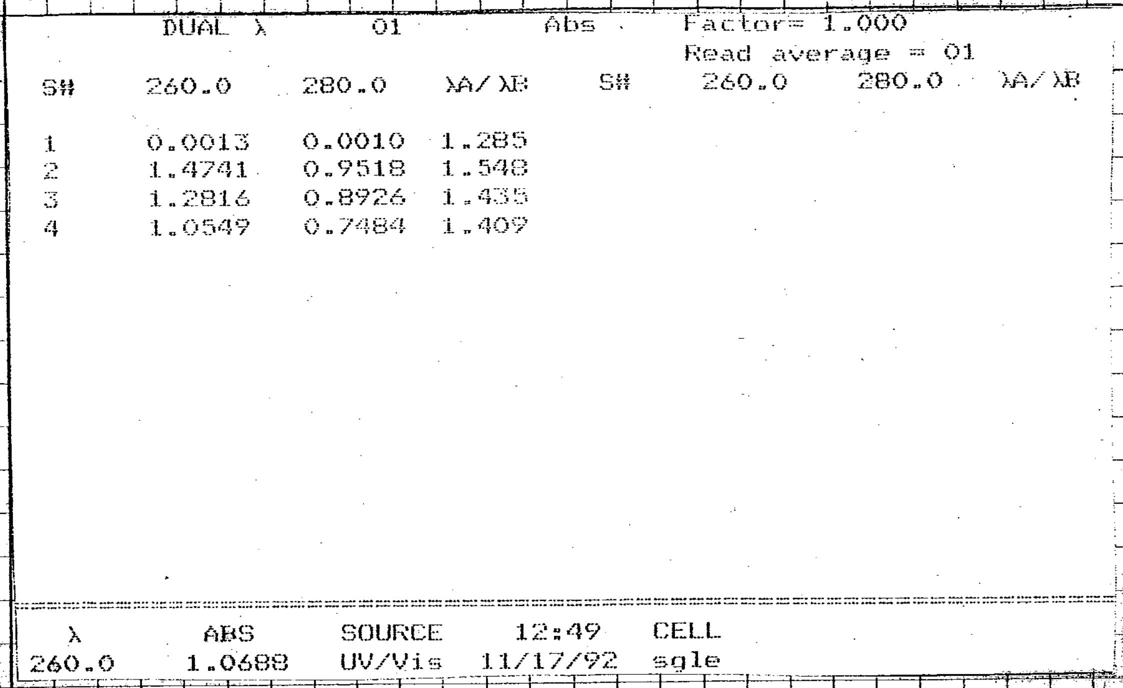


VE3F1
VN0A R1
3



```
        DUAL λ        01          Abs      Factor= 1.000
                                           Read average = 01
 S#    260.0     280.0    λA/λB    S#     260.0     280.0    λA/λB
       50ul sample ,GB, MC, C100
 1     0.0001    0.0001   1.000 Blank- H2O
 2     0.0254    0.0203   1.255 VEE #1
 3     0.0238    0.0189   1.259    #2
 4     0.0176    0.0154   1.140    #3
 5     0.0229    0.0165   1.384   26S
 6     0.0123    0.0104   1.181   GP

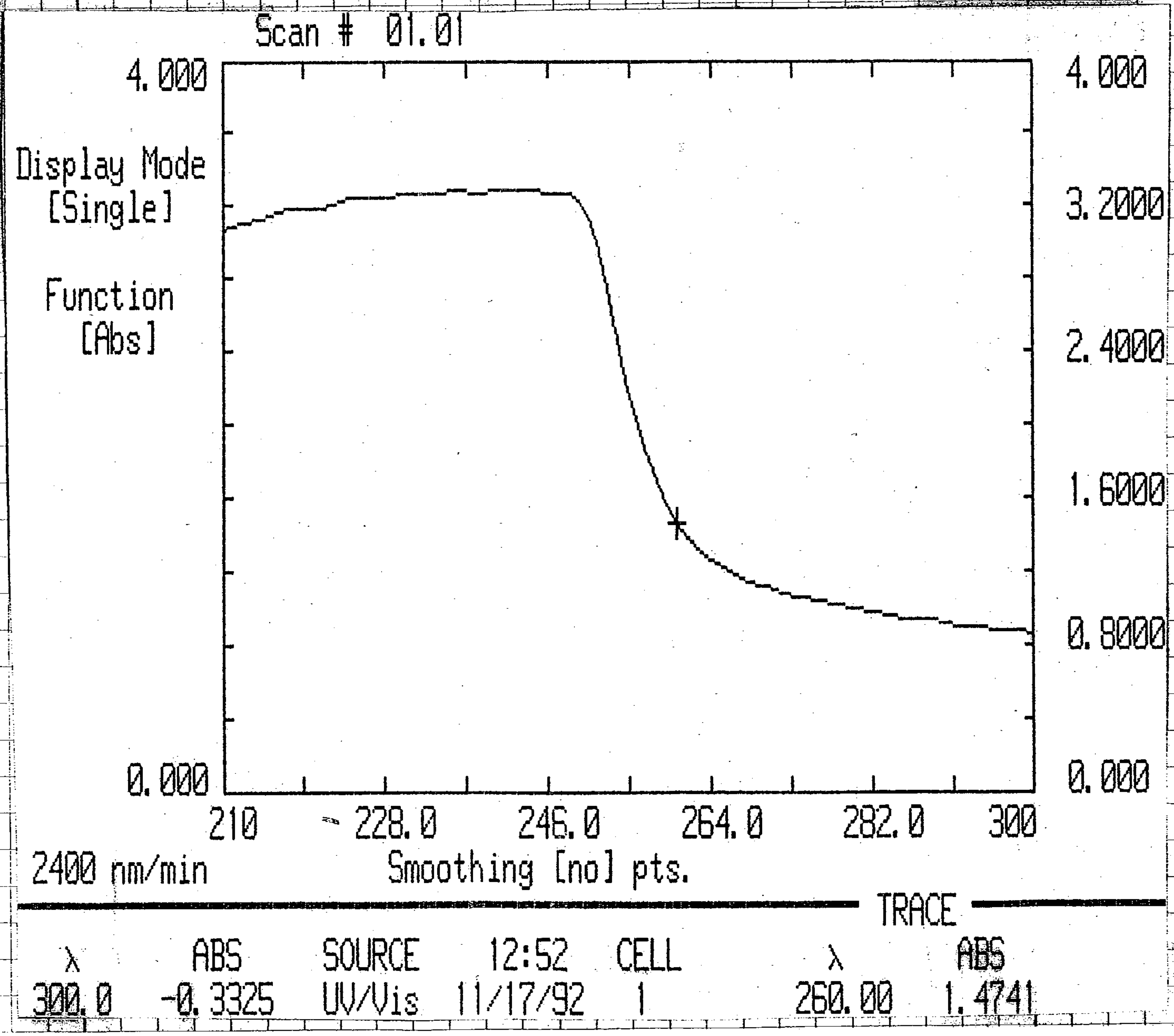
   λ        ABS      SOURCE     10:06    CELL
 260.0     2.3870    UV/Vis   11/17/92   sgle
```



```
          DUAL  λ          01               Abs .     Factor= 1.000
                                                      Read  average = 01
S#       260.0       280.0     λA/λB     S#      260.0       280.0     λA/λB

1        0.0013      0.0010    1.285
2        1.4741      0.9518    1.548
3        1.2816      0.8926    1.435
4        1.0549      0.7484    1.409


 λ          ABS        SOURCE      12:49     CELL
260.0      1.0688      UV/Vis    11/17/92    sgle
```

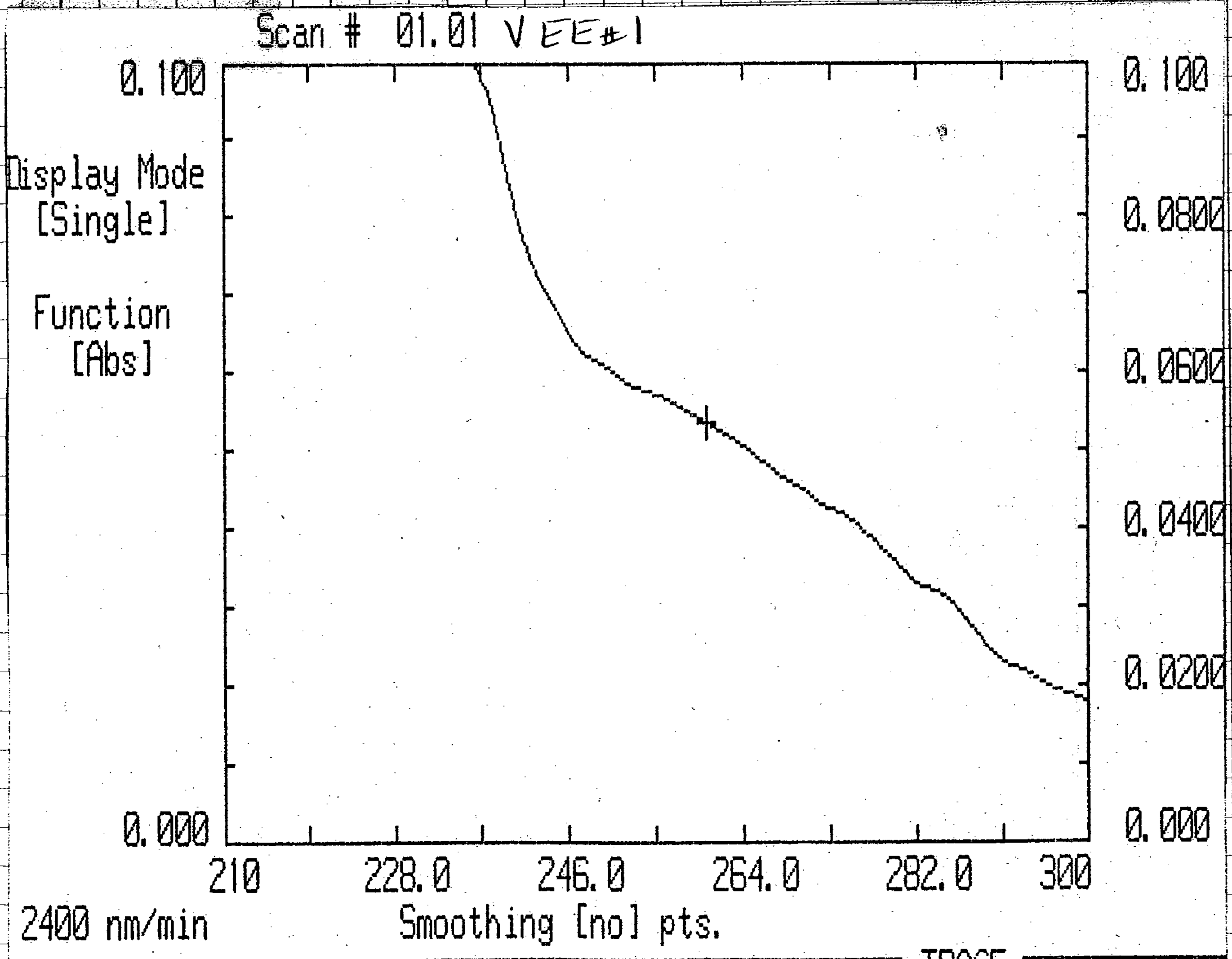


```
  λ        ABS      SOURCE     12:52    CELL          λ        ABS
300.0   -0.3325     UV/Vis   11/17/92    1          260.00    1.4741
```

107

```
        DUAL λ        01          Abs       Factor= 1.000
                                            Read average = 01
SH     260.0     280.0    λA/λB      SH     260.0     280.0    λA/λB
      50ul sample after C10 of filtrate from C100
1      0.0006    0.0005   1.111
2      0.0563    0.0382   1.471 VEE#1
3      0.0427    0.0289   1.476   " #2
4      0.0311    0.0206   1.513   " #3
5      0.0468    0.0316   1.480 265
6      0.0476    0.0311   1.527 GP

  λ         ABS       SOURCE      11:37     CELL
260.0     2.4092     UV/Vis    11/17/92    sgle
```



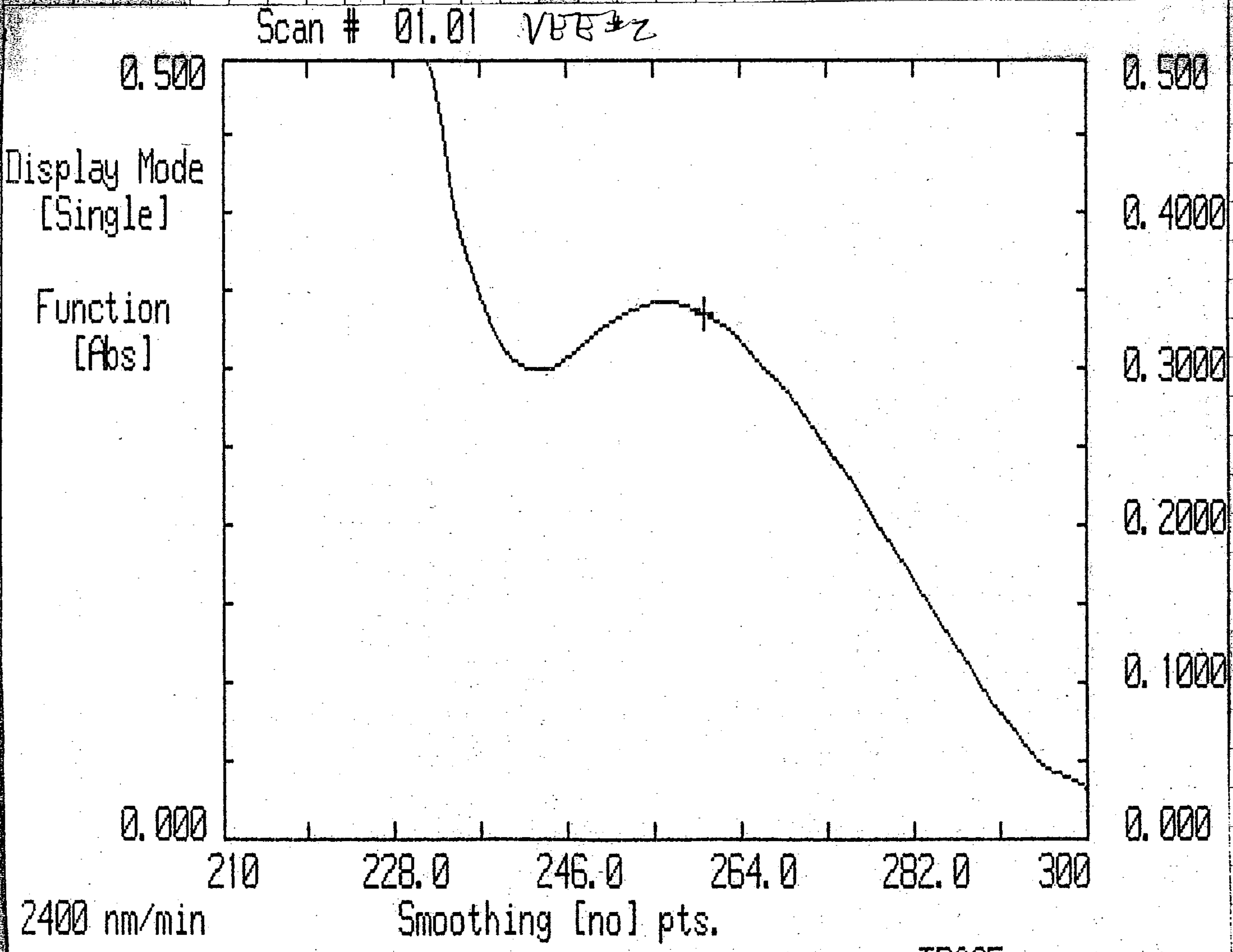
TRACE

λ ABS SOURCE 11:44 CELL λ ABS

```
        DUAL λ        01              Abs        Factor= 1.000
                                                 Read average = 01
S#     260.0       280.0     λA/ λB      S#      260.0     280.0    λA/ λB
    50ul sample   PCR PRODUCT, GLMP agarose, MC, BamHI/HindIII, EtOH ppt
 1     0.0006      0.0000    +****
 2     0.1607      0.1153    1.394  VEE #1
 3     0.3406      0.1944    1.751   VEE#2
 4     0.0531      0.0376    1.414   VEE#3
```

#2 8.035 ug/ml = 0.008035 ug/ul - need 57ul = 0.46 ug
#3 17.03 ug/ml = 0.01703 ug/ul - need 25ul = 0.43 ug
#4 2.655 ug/ml = 0.002655 ug/ul - need 90ul = 0.24 ug

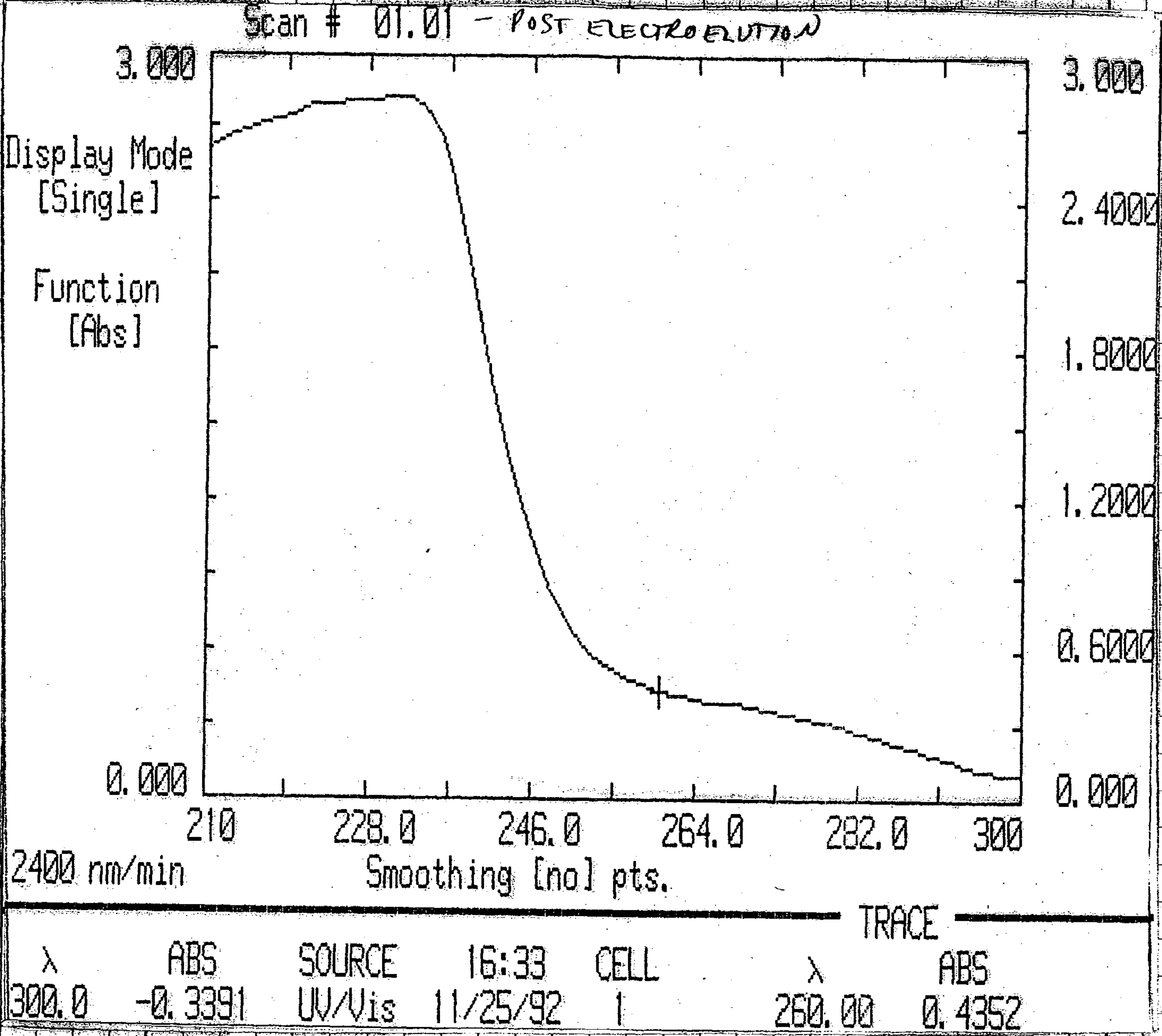
```
  λ          ABS        SOURCE       09:47     CELL
 260.0      0.0522      UV/Vis     11/18/92    sgle
```



TRACE

λ ABS SOURCE 09:51 CELL λ ABS

Scan # 01.01 - POST ELECTROELUTION



Display Mode
[Single]

Function
[Abs]

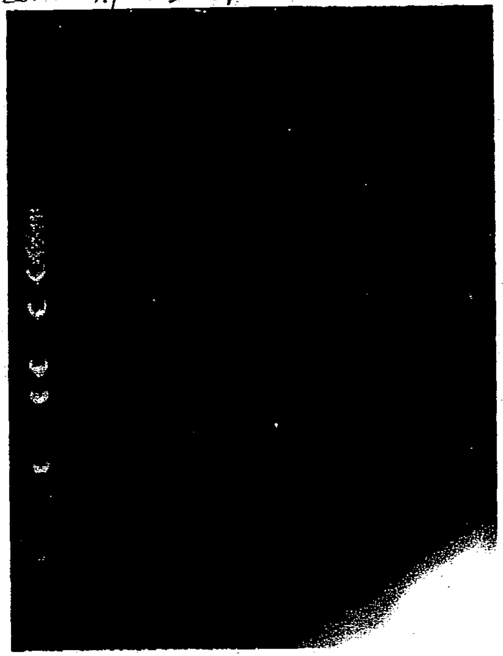
2400 nm/min

Smoothing [no] pts.

TRACE

```
 λ       ABS     SOURCE    16:33   CELL        λ       ABS
300.0  -0.3391   UV/Vis  11/25/92   1       260.00   0.4352
```

ZowAT#11/VE3F1/VE3R2

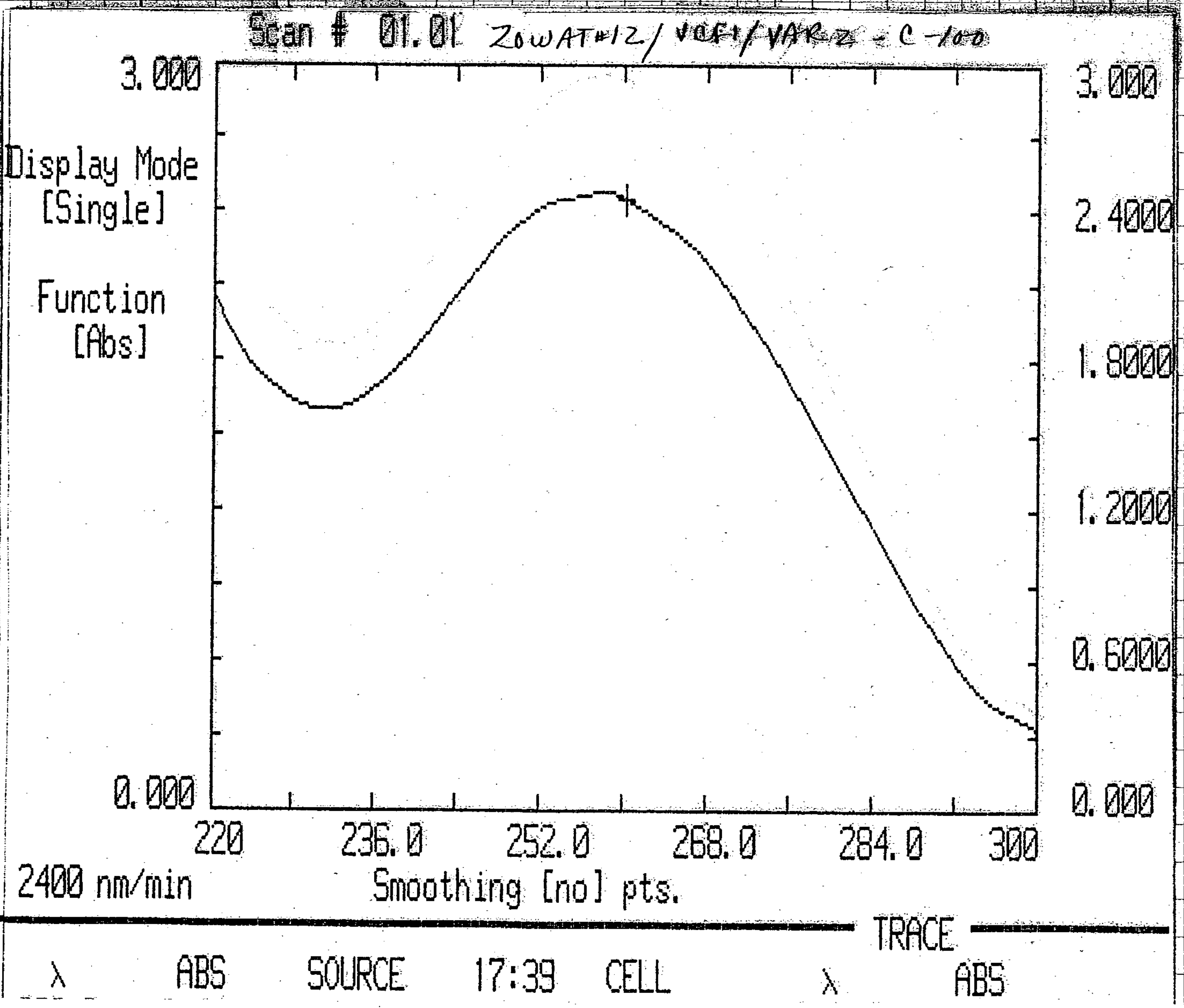


15NOV92

```
        DUAL λ       01            Abs     Factor= 1.000
                                           Read average = 01
 S#      260.0     280.0    λA/λB    S#     260.0     280.0     λA/λB

 1       0.0003    0.0002   1.258
 2       0.4399    0.3047   1.443 [illegible] 11VE3F1/VE3R2 - POST ELECTROELUTION
 3       0.0288    0.0186   1.549         "          - MEMBRANE [illegible]

 λ          ABS       SOURCE     16:29     CELL
260.0      0.0261     UV/Vis   11/25/92    sgle
```

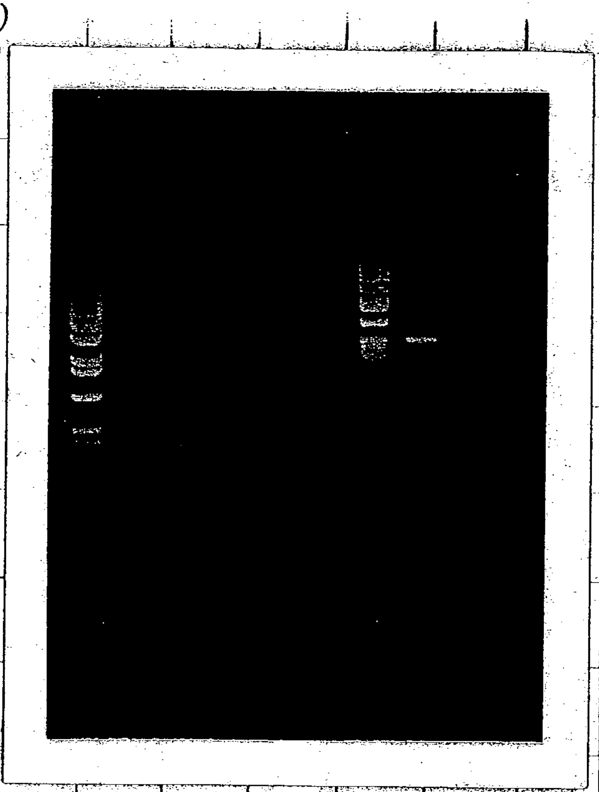


Scan # 01.01 Z0WAT#12/ VCF1/VAR2 - C-100

5X PCR amplification

25Nov92
ZOWAT #12
(3,914) (3095)

	λC	VC-F1 VAR2 1X	5X	VE3-F1 VAR2 1X	5X
H₂O		18.1	393.5	18	390
Template	1	0.5	2.5	0.5	2.5
10X Rxn buffer	I 18ul MM	I 18ul MM	90ul	I 18	90ul
10mM dNTP					
FORWARD PR	5	1.2	6	1.9	9.5
REVERSE PR	5	1.1	5.5	1.1	5.5
	Mix, vortex				
Taq pol	0.5	0.5 21.3	2.5 106.5	0.5 22	2.5 110
TV	100ul				
Mineral oil	100ul				



Thermocycler Program
Step-cycle: 95°C-30sec
52°C-30sec } 30cycles
72°C - 3min
Delay cycle: 72°C for 10min
Soak cycle

Master Mix
dATP = 12x2 = 24ul
dCTP = " 24
dGTP = " 24
dTTP = " 24
10X Rxn buffer (I) = 10 x 12 = 120ul

```
        DUAL λ         01              Abs        Factor= 1.000
                                                  Read average = 01
S#      260.0       280.0      λA/λB      S#      260.0       280.0      λA/λB
        50ul sample
1       0.0000     -0.0001    -0.000
2       2.4705      1.5060     1.640 ZOWAT#12/VCF1/VAR2 -C100
3       1.9538      1.1541     1.692   "  /VE3F1/VAR2 -C100
```

#2 123,525ug/ml = 0.1235ug/ul = 6.2ug in sample
#3

①

PCR 1ST ROUND
AMPLIFICATION
30NOV92

DNA → 5X AMP of 20WAT 12 / VCF1 VAR2 - 25NOV92 MgCl2

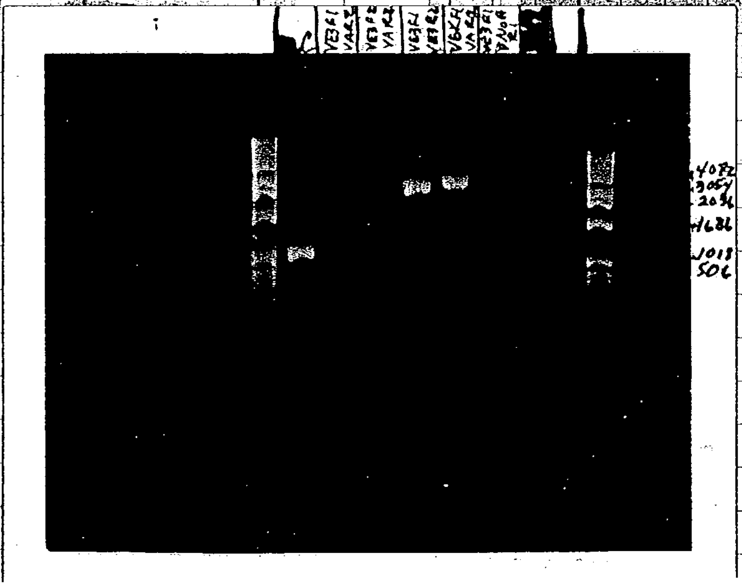
	λC	3,095 VE3-F1 VAR2	3,090 VE3-F2 VAR2	1,491 VE3F1 VE3-R2	1,585 V6K-F1 VA-R2	2,868 VE3-F1 VNoAR1	3,914 VCF1 VAR2
H_2O	70.5	76.5	76.8	75.9	77.3	76.3	77.2
Template	1	2ul	2ul	2ul	2ul	2ul	2ul
FORWARD PR	5	1.9	1.6	1.9	1.1	1.9	1.2
REVERSE PR	5	1.1	1.1	1.7	1.1	1.3	1.1
10X RXN (I) BUFFER }	18ul MM						
10mM dNTP }							
Taq pol (ul)	0.5						
TV	100						

Note: This PCR used a template DNA from a 5X amplification that had not been cleaned up

24.5
23.5
23.2
24.1
22.7
23.7
22.8

Master Mix: dATP 8x2=16
dCTP "
dGTP "
dTTP "
Reaction Buffer (I) 8x10 = 80
144

Thermocycler Program:
Step cycle: 30 sec @ 95°C } 30 cycles
30 sec @ 52°C
3 min 72°C
Delay cycle: 10 min @ 72°C
Soak cycle: 4°C

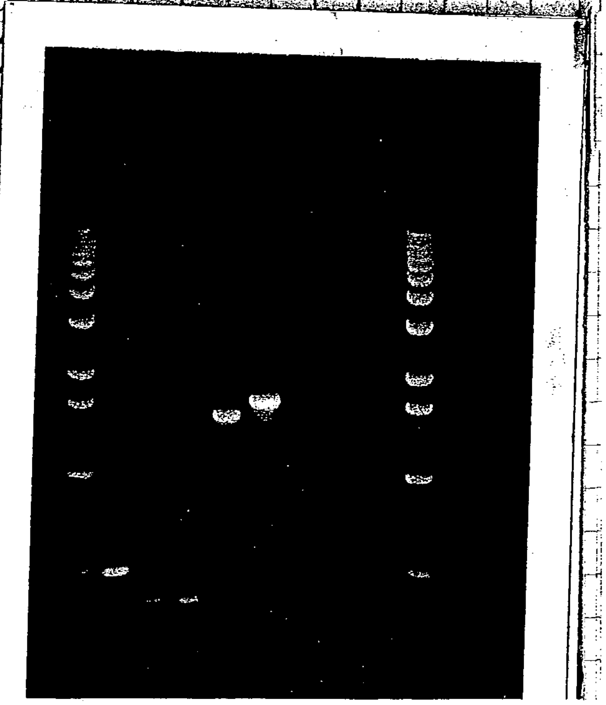


PCR 1st ROUND AMPLIFICATION

DNA →	λ C	5X AMP. of ZOWAT#12/Nc f VARZ-25NOV92 - $\boxed{C100}$ /					
EXPECTED LENGTH (bp)	500	3,095	3,090	1,491	1,585	2,868	3,914
		VE3-F1	VE3-F2	VE3F1	V6K-F1	VE3F1	VC F1
		VARZ	VARZ	VE3R2	VA-R2	VN1AR1	VARZ
H2O (ul)	70.5	78	78.3	77.4	78.8	77.8	78.1
DNA template (ul)	1	0.5	0.5	0.5	0.5	0.5	0.5
FORWARD PR (ul)	5	1.9	1.6	1.9	1.1	1.9	1.2
REVERSE PR (ul)	5	1.1	1.1	1.7	1.1	1.3	1.1
10X RXN BUFFER (I)	18ul M.M						
10mM dNTP							
	Vortex, microfuge						
Taq pol (ul)	0.5 19.5	0.5 22	0.5 21.7	0.5 22.6	0.5 21.2	0.5 22.2	0.5 21.3
TV (ul)	100	100	100	100	100	100	100
Mineral oil (ul)	100ul →						

Thermocycler Program:
Step cycle 30 cycles {30 sec @ 95°C, 30 sec @ 52°C, 3 min @ 72°C}
Delay cycle: 10 min @ 72°C
Soak cycle: 4°C

Master Mix
Rxn buffer I: 8 × 10 = 80 ul
dATP 8 × 2 = 16u
dCTP " 16u
dGTP " 16u
dTTP " 16ul



```
        DUAL λ      01         Abs     Factor= 1.000
                                       Read average = 01
S#    260.0    280.0    λA/λB    S#    260.0    280.0    λA/λB
 5ul in 500ul H2O =  1:101 dil  200ul total sample
1      0.0001  -0.0001 -2.327
2     -0.0520  -0.0433  1.201
3     -0.0520  -0.0433  1.201
4     -0.0006  -0.0007  .8000  - Blank (H2O)
5      0.1537   0.0754  2.039 VARZ
6      0.0834   0.0441  1.890 VE3F1

==========================================================
 λ        ABS      SOURCE     14:05    CELL
260.0   -0.0005     UV      12/02/92    1
```

```
                    - 394 Synthesis Setup Listing -        (Version 1.01)

Column 4                                                  10:44:52A ,
```

~~[illegible]~~ 10EC92

2/92

```
Run ID   :
Cycle    : Cyc02 user
End Proc: End CESS       (DMT - On )
Sequence: VE3F1

Total bases -  44
        A-  9, G- 10, C- 16, T-  9, 5-  0, 6-  0, 7-  0, 8-  0
        (mixed bases-  0)

MW:  14348.8

     5'> GGT CTA GGA TCC GCC GCC ACC ATG GCT TCA CTA GTG ACC

         ACC AT  <3'
```

Sequ

$Em = A(16000) + C(7000) + G(12000) + T(9600)$

$= 9(16000) + 16(7000) + 10(12000) + 9(9600)$

$= 144{,}000 + 112{,}000 + 120{,}000 + 86{,}400$

$Em = 462{,}400$

Molarity $= \dfrac{OD_{260} \cdot \text{Dil factor}}{Em}$

$= \dfrac{0.0834 \cdot 101}{462{,}400}$

$= 0.0000182$

Molarity $= 18.2$ pmol

PCR: Need 100 pmol/ul

use 5.5 ul/rxn

```
                    - 394 Synthesis Setup Listing -        (Version 1.01)

Column 2                                                  10:44:49A ,
```

~~[illegible]~~ 10EC92

/92

```
Run ID   :
Cycle    : Cyc02 user
End Proc: End CESS       (DMT - On )
Sequence: VAR2

Total bases -  34
        A- 14, G-  5, C-  7, T-  8, 5-  0, 6-  0, 7-  0, 8-  0
        (mixed bases-  0)

MW:  11277.8

     5'> CTC GGC TTT CGA AGA ATA TTA AAA ACA AAA TCC G   <3'
```

Sequ

$Em = A(16000) + C(7000) + G(12000) + T(9600)$

$= 14(16000) + 7(7000) + 5(12000) + 6(9600)$

$= 224{,}000 + 49{,}000 + 60{,}000 + 57{,}600$

$Em = 390{,}600$

Molarity $= \dfrac{OD_{260} \cdot \text{Dil factor}}{Em}$

$= \dfrac{0.1537 \cdot 101}{390{,}600}$

$= 0.0000397$

$= 39.7$

PCR: Need 100 pmol/ul

use 2.5 ul/rxn

Protocol for Ampliwax PCR Gem-Mediated Hot Start PCR

1. Calculate the number of amplification reactions to be done, including the positive and negative control. Prepare a small excess of Lower Reagent Mix over that needed for the planned number of reactants, to account for handling losses.

Component	Volume/Reaction	Final Concentration
10X PCR Buffer II	2.5 ul	1.25X
$MgCl_2$ Solution, 25 mM	10 ul	2.5 mM
dATP, 10 mM	2 ul	200 mM
dCTP, 10 mM	2 ul	200 mM
dGTP, 10 mM	2 ul	200 mM
dTTP, 10 mM	2 ul	200 mM

Add 20.5 ul to each reaction tube.
Add primers and QS to 25 ul w/ autoclaved deionized ultrafiltered water.

Preferably the Lower Reagent Mix should not include carrier protein. Mix the bulk lower reagent mix by vortexing. ~~Aliquot 25 20.5 ul into~~ Be careful to avoid splashing liquid onto the tube wall. If any liquid is present on the wall of a tube, tap the tube gently on a bench top for a few seconds to move all droplets into the aqueous layer before forming the wax layer.

Carefully add one Ampliwax PCR gem 100 to each tube. In order to transfer one PCR gem into each reaction tube, first pour a small number of PCR gems into a clean weighing boat. Then, while holding a corner of the weighing boat over the top of the open tube, use a clean spatula to push ~~apart~~ one PCR gem into the tube

~~Melt the wax by incubating the reaction tubes at 75 to 80°C for 5 to 10 min in a lab oven or dry well. After this layering step let the tubes cool to room temperature~~
Wax may be layered in a thermocycler. In this case reaction tubes must be tightly capped and care should be taken to prevent transfer of possible DNA or PCR product contamination on the outside of the tube to the inner surface of the reaction tube.
For the DNA Thermocycler
Step-cycle: 5 min @ 80°C } 1 cycle
1 min @ 25°C }

Upper Reagent Mix Preparation:

dNTPs
8024
7/93

PCR w/ AMPLIWAX

DNA →	λC	5 X AMP of ZOWAT #12/ VCF1/VAR2 - 25NOV92-C100			
EXPECTED LENGTH (bp)	500	3,095	3,090	2,868	3,914
used VAR2 of	λC	VE3-F1 VAR2 1	VE3-F2 VAR2 2	VE3F1 VNoAR1 3	VCF1 VAR2 4
Master Rgt Mix (ul)	20.5	20.5	20.5	20.5	20.5
Forward pr (ul)	5	5.5	1.6	5.5	1.2
Reverse pr (ul)	5	1.1	1.1	1.3	1.1
H_2O (TV = 30 ul)	—	2.9	6.8	0.7	7.2

Vortex, microfuge

Add 1 PCR gem / vial

Thermocycler Program:
Step cycle : 5 min @ 80°C
1 min @ 25°C
> User 464 file 38

Add:

10X PCR buffer II	10	10	10	10	10
Taq pol	0.5	0.5	0.5	0.5	0.5
DNA template dilution	until 1	59.5	59.5	59.5	59.5

100.0
-40.5
59.5
×5
297.5
-2.5
295.0

Master Rgt Mix:

10X PCR Buffer II 6 x 2.5 = 15
$MgCl_2$ Solution 6 x 10 = 60
dATP 6 x 2 = 12
dCTP 6 x 2 = 12
dGTP 6 x 2 = 12
dTTP 6 x 2 = 12
123

Thermocycler Program
Stepcycle : 95°C - 1 min
52°C - 30 sec
72°C - 3 min
Delay: 72°C - 10 min
Soak : 4°C

DNA template dilution
2.5 ul template + 295 ul H_2O

Component	Volume/ reaction	Final Concentration
10X PCR buffer II	10 ul	1.25X
AmpliTaq DNA Pol	0.5 ul	2.5 U/rxn
DNA template		
H_2O to 75 ul		

~~Add the appropriate volume~~ Add the upper reservoir above the solid wax layer in each tube.

Thermocycler Program

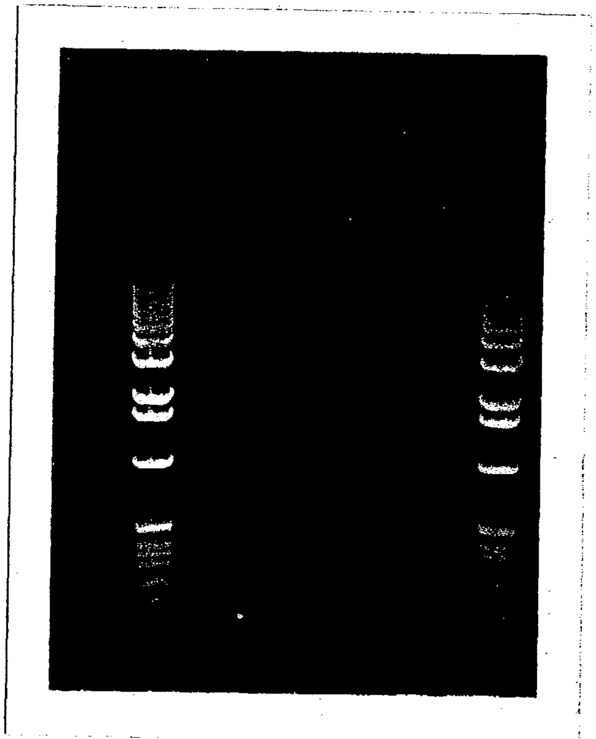
Step-cycle: 95°C - 1 min

52°C - ~~1 min~~ 30 sec

72°C - 3 min

Delay: 72°C - 10 min

Soak: 4°



3Dec92 Have 5 mCi ^{35}S-cysteine (NEG-022T) Lot 2869-233

12:20P in 0.453 ml of 10 mM PDT = 11 uCi/ul

Label at 25 uCi / T25 in 5 ml = 125 uCi total = 11.4 ul of ^{35}S-cysteine in 25 ml of Grace's media + 1% GLUT, gentamicin, fungizone.

3Dec92 FA set up 30 Nov 92. Aspirated media. Air dry 10 min.

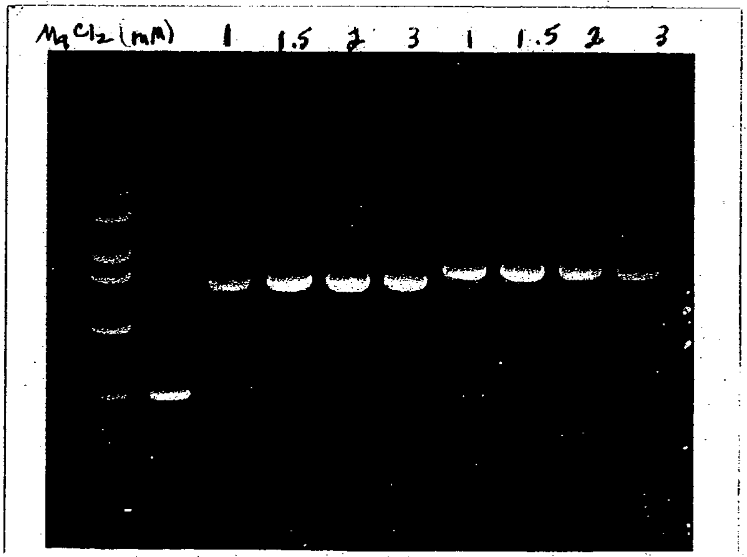
Acetone fix 4 min. Store -20°C

MgCl2 Optimization
2 Dec 92

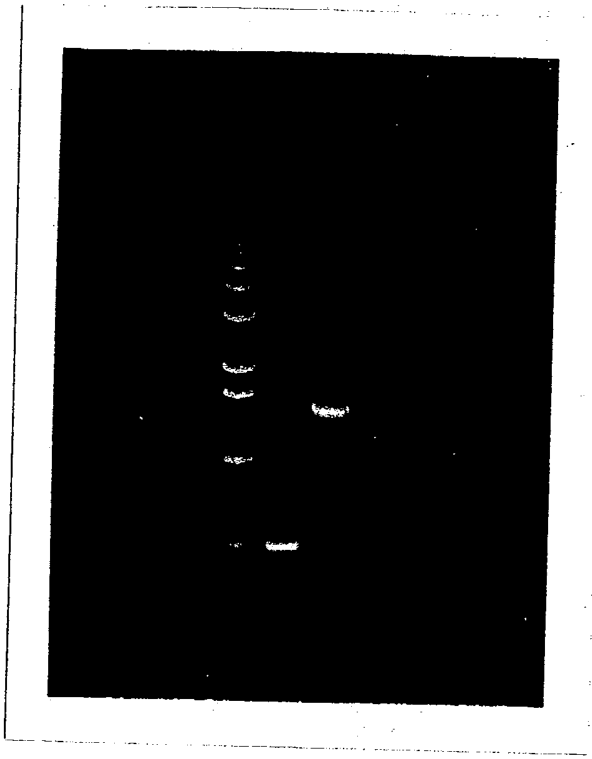
DNA →	λC	5X AMP of ZOWAT #12 / VC F1/VAR2 - 25NOV92 / C100							
EXPECTED LENGTH	500	VE3-F1/VE3.R2 (1,491 kb)				V6K-F1/VAR2 (1,585 kb)			
[MgCl2] mM		1	1.5	2	3	1	1.5	2	3
H2O (ul)	70.5	22.2 72.8	29.2 70.8	31.2 68.8	35.2 64.8	29.6 70.4	31.6 68.4	33.6 66.4	37.6 62.4
Template (ul)	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Forward PR (ul)	5	2.5	2.5	2.5	2.5	1.1	1.1	1.1	1.1
REVERSE PR (ul)	5	1.7	1.7	1.7	1.7	5.5	5.5	5.5	5.5
10x Rxn buffer	(I) 10	18 ul MM (II)		II					
10mM dNTP	2ul each dNTP								
25 mM MgCl2	—	4	6	8	12	4	6	8	12
	Vortex, microfuge								
Taq pol (ul)	0.5								
	Vortex, microfuge								
Mineral oil	100ul								

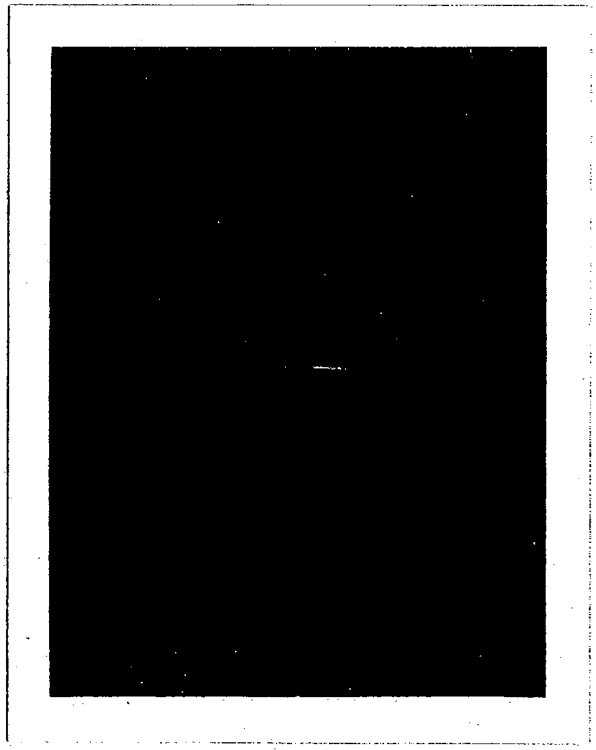
Thermocycler program
Step-cycle - File 40
95°C - 30 sec
52°C - 30 sec
72°C - 3 min
} 30 cycles
Delay = 72°C - 10 min
Soak = 4°C

Master Mix
10X Rxn buffer II = 9x10 = 90 ul
d ATP = 2x9 = 18 ul
d GTP = " 18 ul
d CTP = " 18 ul
d TTP = " 18 ul
TV = 162 ul

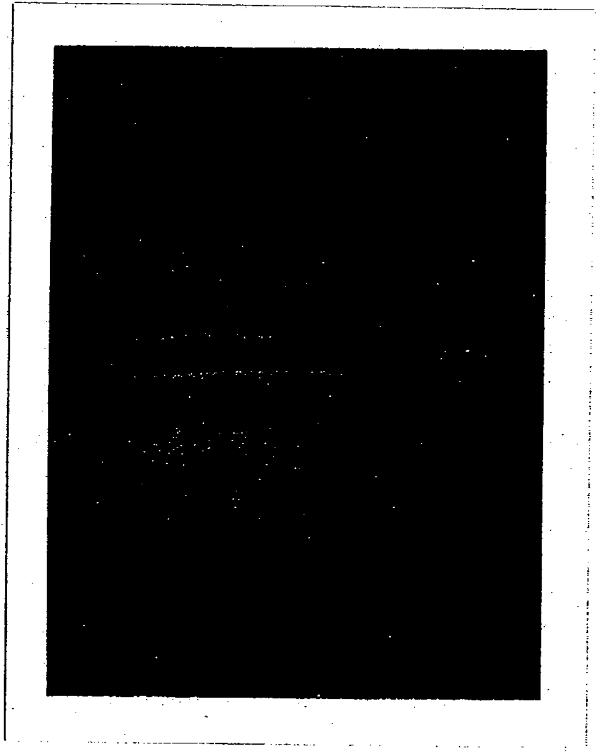


0.9% LE agarose gel in 1XTAE





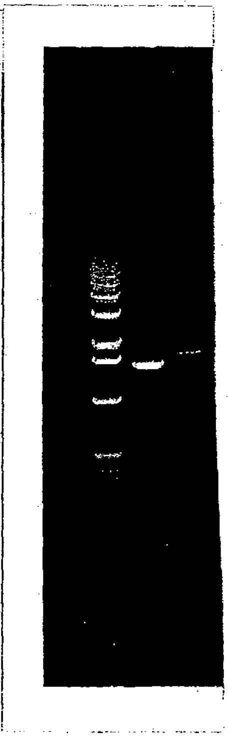
VE3 F1
VE3 R2



VGK F1
V4R2

5X PCR AMPLIFICATION
3DEC92

DNA	xC	5X AMP of ZOWAT #12 VCF1/VAR2 - 25NOV92 - UD				
EXPECTED LENGTH (bp)	500		1491 Kb VE3-F1/VE3R2		1585 Kb V6K F1/VAR2	
[MgCl2]			1X (2)	5X	1X (1.5)	5X
H2O			68.8	344	68.4	342
Template (ul)	1		0.5	2.5	0.5	2.5
forward pr (ul)	5		2.5	12.5	1.1	5.5
Reverse pr (ul)	5		1.7	8.5	5.5	27.5
10X Rxn buffer	I	MM II	18	90	18	90
10mM dNTP	2ul ea dNTP					
25mM MgCl2			8	40	6	30
	Vortex, microfuge					
Taq pol	0.5		0.5	2.5	0.5	2.5
	Vortex, microfuge		31.2	156		158
Mineral oil	100ul					
Thermocycler program Step cycle 95°C	-30 sec	Master Mix (II) 10X Rxn buffer II = 12x10 = 120ul				
Delay - 72° Soak 4°						



```
        DUAL λ        ·1           Abs       Factor= 1.000
                                             Read average = 01
S#      260.0      280.0      λA/λB      S#      260.0      280.0      λA/λB
1       0.0044     0.0028     1.564
2       0.0002     0.0003     .8516
3       2.6704     1.6838     1.5860
4       0.6744     0.4184     1.611
```

GB, GC, QD

~~0.0044 0.0028 1.564~~

2 blank

3 DNA PREP ZOWAT12 VE3F1/VE3R2

4 " " V6KF1/VAR2

#3 133.52ug/ml = 0.13352ug/ul = 6.7ug in sample

#4 33.72ug/ml = 0.03372ug/ul = 1.7ug in sample

8

Molecule Name: 26X 3911 BPS
Region Identified: Start @ bp 1 End @ bp 3911

Report Date: 12/03/92

PCR Primer Design Report

---- LEFT PRIMER ---- V26M-F1

M13

Sequence: 5'-GTAAAACGACGGCCAGTCATAGTCTAGTCCGCCAAG-3'

Length: 36 Bp-pos: 12 5' extension: GTAAAACGACGGCCAGT 17
%GC: 52; Tm C: 63; Composition: A:11 C:10 G:9 T:6

Criteria: % GC: Min 50, Max 60; Tm C: Min 55, Max 80
Energy cutoff: 0.0 kcal; Reject runs >= 3 bases
Meets criteria selected: NO

---- RIGHT PRIMER ---- V26M-R1

WRONG SEQ - REPEATED 11 Dec 92 See p 123 this notebook

Sequence: 5'-CAGGAAACAGCTATGACAGTTCTATGTAAGCAGCTT-3'

Length: 36 Bp-pos: 3831 5' extension: CAGGAAACAGCTATGAC
%GC: 41; Tm C: 57; Composition: A:12 C:7 G:8 T:9

Criteria: % GC: Min 50, Max 60; Tm C: Min 55, Max 80
Energy cutoff: 0.0 kcal; Reject runs >= 3 bases
Meets criteria selected: NO

[illegible] 21553

509 Oakmont Dr
Frederick
Eastview
Purchased 1977
assessment $72,000

Primer Balance:

	Left	Right	Comment
Length	36	36	
% GC	52	41	11% difference
Tm C	63	57	6C difference

Primer Cautions:

	Left	Right	
3 or more G or C at 3' end	NO	NO	
Runs of bases	YES	YES	(see criteria for
Secondary structure	NO	NO	(primers, above

Primer Interactions:

(Number of adjacent bases in largest duplex)

	L:L	R:R	L:R
Any Primer-dimers	4	4	7
3'end involved	2	4	3

*** See Page 2 for duplex structures ***

1)

```
- 394 Synthesis Setup Listing -        (Version 1.01)01)92

Column 1                                   4:47:16P ,12/ 2/92/92

Run ID  :
Cycle   : Cyc02 user
End Proc: End CESS      (DMT - On )
Sequence: M13

Total bases -  17
        A-  6, G-  5, C-  4, T-  2, 5-  0, 6-  0, 7-  0, 8-  0
        (mixed bases-  0)

MW:   5666.4

     5'> GTA AAA CGA CGG CCA GT  <3'
```

Em= A(16000) + C(7000) + G(12000) + T(9600)

6(16000) + 4(7000) + 5(12000) + 2(9600)

96,000 + 28,000 + 60,000 + 19,200

Em = 203,200

$$\text{Molarity} = \frac{OD_{260} \times \text{dil. factor}}{EM}$$

$$= \frac{0.1577 \times 101}{203,200} = \frac{15.9277}{203200}$$

$$=$$

78.3 pmol

PCR need 100pmol/µl $\frac{100}{78.3} = 1.27$

use 1.27µl/rxn

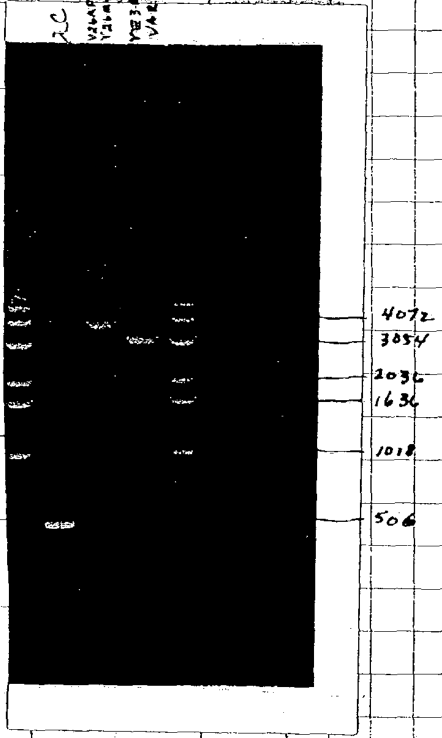
```
        DUAL  λ        01           Abs      Factor= 1.000
                                             Read average = 01
S#     260.0      280.0    λA/λB     S#     260.0     280.0    λA/λB
      5µl in 500µl H2O = 1:101 dil        200µl total sample
1     -0.0006    -0.0003   2.000
2      0.3050     0.2096   1.455 V26MF1   4DEC92
3      0.2887     0.1836   1.572 V26MR1     "
4      0.1577     0.1052   1.498 M13        "
5      0.1993     0.1410   1.413 VE3-F1     "


 λ        ABS       SOURCE     15:29     CELL
260.0   -0.0005    UV/Vis   12/04/92     1
```

1st ROUND PCR

ZOWAT 12

		3,095, VE3F1 VAR2	3,853, V26MF1 V26MR1
H2O	10.5	78.4	78.2
DNA template	0.1	0.5	0.5
FORWARD PR	5	1.5	1.3
REVERSE PR	5	1.1	1.5
10X RXN BUFF.(I)	} 8ul MM	⟶	
10mM dNTP			
Taq pol	0.5	0.5	0.5
		21.6	21.8



3831
−12
3819
+17
3 +17
3853

28
1
0.5
29.5

100

Thermocycler
Step cycle 30 sec 95
30 sec 52
3 min 72
Delay

Master Mix
Rxn buffer I 4×10=40
dNTP 4×2=8
8
8
8

GIBCO BRL

LIFE TECHNOLOGIES, INC.
GAITHERSBURG, MARYLAND 20898

8011SA
Lot No. CDP109
Bacterial Alkaline Phosphatase

2,500 units; 150 U/µl
Store at -20°C (not frost-free)

Description:
Bacterial Alkaline Phosphatase (BAP) is a phosphomono-esterase which hydrolyzes 3' and 5' phosphates from DNA and RNA. It is suitable for removing 5' phosphates prior to end-labeling and for dephosphorylating vectors prior to insert ligation. BAP is active at 65°C for at least 1 h and can be inactivated by phenol extraction. It is sensitive to inhibition by micromolar amounts of inorganic phosphate. The enzyme is purified from E. coli C90.

Unit Definition:
One unit hydrolyzes 1 nmol of ATP in 30 min at 37°C.

Storage Buffer:
10mM Tris-HCl (pH 8.0)
120 mM NaCl
50% (v/v) glycerol

Quality Control Assays:
This product has passed the following quality control assays: absence of detectable endodeoxyribonuclease, exodeoxyribonuclease and ribonuclease activities; performance in dephosphorylating blunt-ended DNA. For further details on lot-specific data, please call BRL Technical Services.

Effective 02/08/92

FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC
OR THERAPEUTIC USE IN HUMANS OR ANIMALS.

50mM x10 = 0.5M

1 Dec 92 R.E. w/ BamHI + HindIII

Z12/VE3F1/VE3R2 = 0.13352 ug/ul = 37.4 ul for 5 ug.

Z12/V6KF1/VAR2 = 0.03372 ug/ul - Have ~38 ul left - use entire ~~tube~~ amount.

pBBIII - Invitrogen - Mike Parker used 200 ng - remainder used in this experiment. Added 50 ul H_2O. Transferred 40 ul to tube for digestion

	Z12/VE3F1/VE3R2	Z12/V6KF1/VAR2	pBBIII
DNA	37 ul	38 ul	40 ul
React 3	10 ul	10 ul	10 ul
BamHI	5 ul	5 ul	5 ul
H_2O	48 ul	47 ul	45 ul

Incubate 2H @ 37°C.
add: 0.1 ul of 5M NaCl
5 ul of HindIII to each tube
Incubate: 2H @ 37°C
Heat 65°C for 20 min. Quench on ice for 10 min.
Extract 1X w/ ϕ/$CHCl_3$/isoamyl alcohol (25:24:1). Vortex 1 min. Microfuge 12,000 x g for 2 min. Back extract w/ 100 mM TRIS-HCl, pH 8.0. $CHCl_3$ extract 2X.
Precipitate w/ 1/10 volume of 3M NaOAc, pH 5.2 and 2 volumes EtOH.
Freeze on dry ice for 15 min. Microfuge 15 min @ 4°C. Wash pellet w/ 100 ul 80% EtOH. Freeze on dry ice for 5 min. Microfuge for 5 min @ 4°C.
Resuspend the ~~DNA~~ SEE insert w/ H_2O to 500 ul. Heat for 10 min @ 45°C. Concentrate and remove the cut ends with a Centricon 100 @ 20°C Centrifuge for ~~30~~ 5 min in a Sorvall SS-34 rotor. ~~Inv~~ Invert filtrate assembly and centrifuge at 2500 rpm for 2 min.
Quantitate $A_{260/280}$. See p 134 for results, including scans.

8 DEC 92 7:10A pBBIII: Resuspend in $\overset{88}{90}$ ul of sterile H_2O ~~on~~, add 10 ul 500 mM Tris HCl, pH 8.0 and 2 ul BAP (150 U/ml) Incubate ~~30 min~~ 1H @ 65°C. Stop reaction by adding 1 ul of 0.5 M EDTA.
Phenol extract 1X, $CHCl_3$ extract 1X, EtOH precipitate as above. Quantitate $A_{260/280}$ - See p 135 for results

[illegible]

8 DEC 92

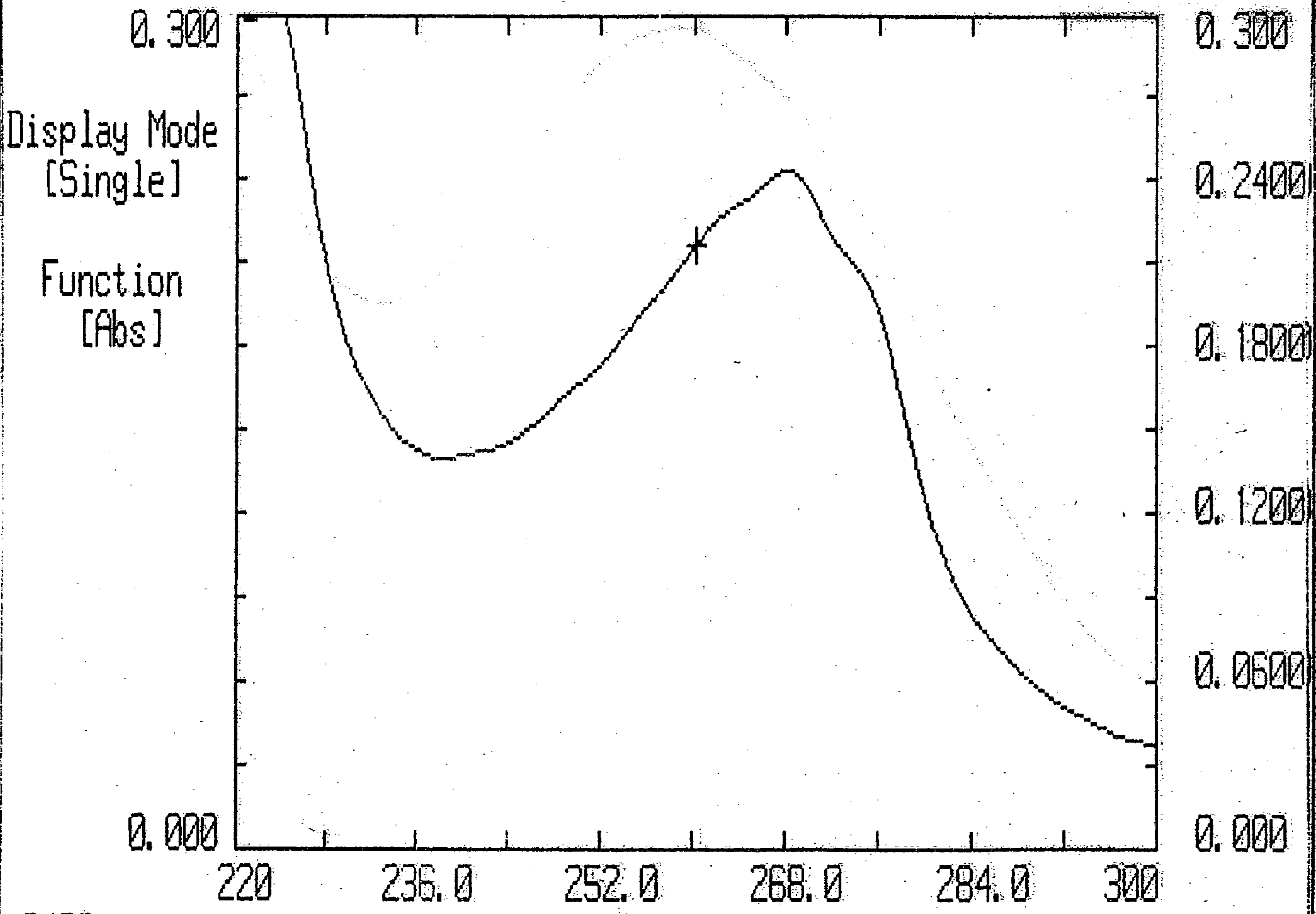
```
          DUAL λ       01          Abs      Factor= 1.000
DNA PCR PREPS: 20WAT12, GB, GC.            Read average = 01
S#     260.0     280.0    λA/λB    S#    260.0     280.0    λA/λB
Cut w/BamHI, HINDIII, C100 ~ 50ul sample
1      0.0011    0.0004   2.572
2      0.2151    0.1413   1.522 V6KF1/VAR2
3      1.3874    0.8585   1.616 VE3F1/VE3R2
```

#2 10.155 ug/ml = 0.011 ug/ul = ~0.54 ug (total sample)
#3 69.31 ug/ml = 0.07 ug/ul = 3.47 ug (total sample)

```
  λ        ABS      SOURCE     08:11    CELL
260.0     2.3245    UV/Vis   12/08/92   sgle
```

10
7.3

Scan # 01.01 V6KF1/VAR2



```
2400 nm/min          Smoothing [no] pts.
                                              TRACE
  λ        ABS      SOURCE     08:14    CELL          λ        ABS
```

```
       DUAL λ      01          Abs        Factor= 1.000
                                          Read average = 01
S#     260.0      280.0     λA/λB     S#     260.0     280.0     λA/λB
       50ul sample
1      0.0003     0.0000    6.243  Blank
2      2.1628     1.2139    1.781  pBBIII/BamHI/HINDIII/
                                   BAP
```

#2 ~~0.417 ug/ml~~ = 108.14 ug/ml = 0.108 ug/ul = 5.4 ug in total sample.

See p 136 for scan

```
  λ          ABS       SOURCE       10:30      CELL
 260.0      2.1672     UV/Vis     12/08/92     sgle
```

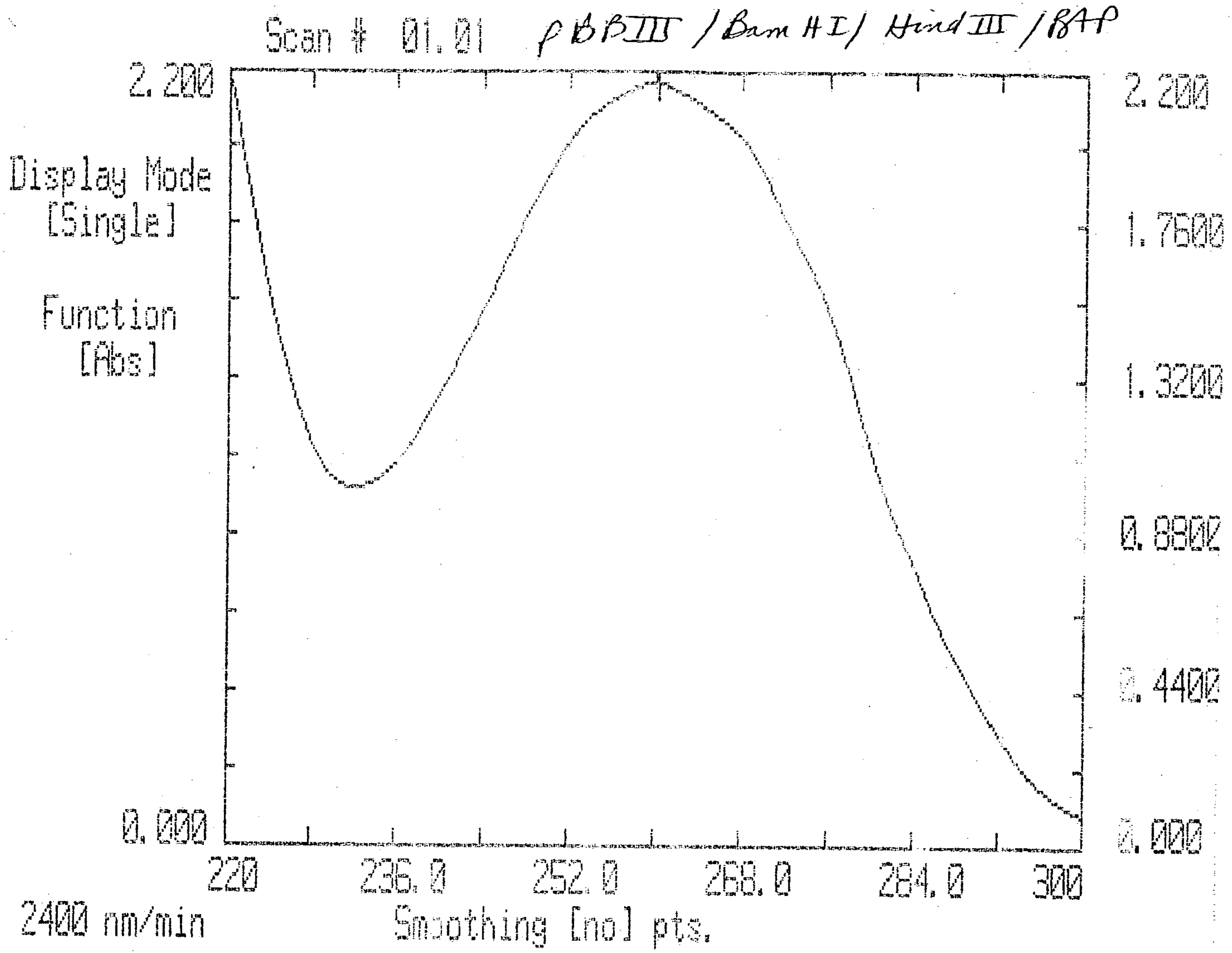
8Dec92	Ligation#	H_2O	10X ligation Buffer	(0.25 ug) pBBIII/ BamHI/ HindIII/BAP	V6RF4/ VAR2	~~Vitamin~~ 20WAT12 VE3F1/ VE3R2	T4 DNA LIGASE
10:00A	1	2.7	1	2.3	3 (0.5 ug)	—	1
	2	2.1	1	2.3	— 3.6 ul of 0.07 ug/ul V:I = 1:1 (0.25 ug)	↙	1
	3	3.2	1	2.3	—	2.5 ul of 0.2 ug/ul V:I = 1:2 (0.5 ug)	1
	4	1.9	1	2.3	—	3.8 ul of 0.2 ug/ul V:I = 1:3 (0.75 ug)	1
	5	5.7	1	2.3	—	—	1

Inc @ 26°C for 4H

2:35P Added 5 ul to 100 ul TOP10F' competent cells.

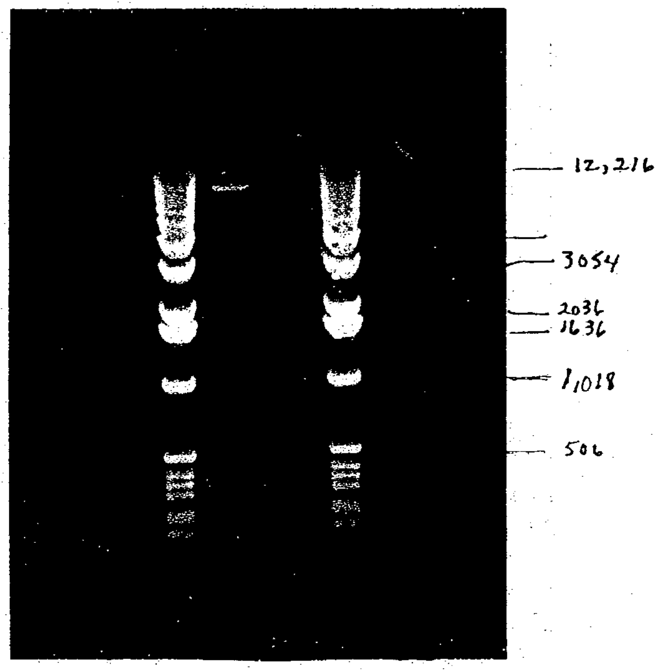
9Dec92 No colonies on any of the plates

Scan # 01.01 pBBIII / Bam HI / Hind III / BAP



TRACE

λ	ABS	SOURCE	10:32	CELL	λ	ABS
300.0	-0.3590	UV/Vis	12/08/92	1	260.00	2.697



1st ROUND PCR AMPLIFICATION
1DEC92

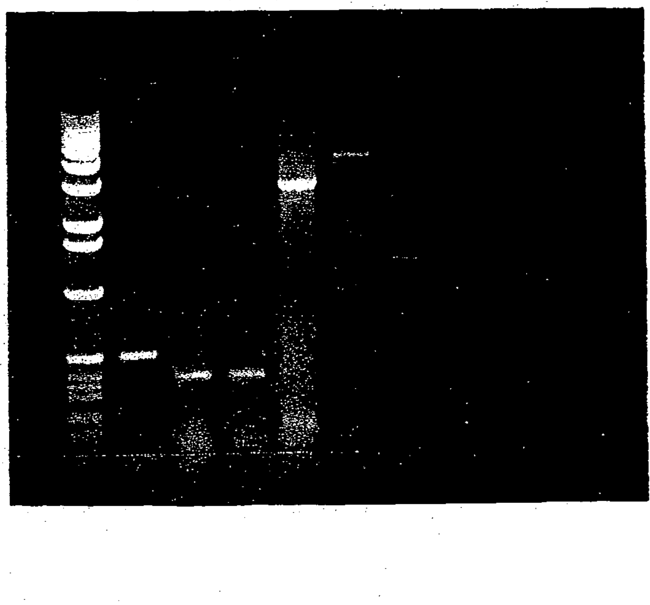
DNA → λC 20WAT12/VE3F1/VAR2

				2,868		
FORWARD PR→	Pr#1	VE3F1	VE3F2	VE3F1	VCF1	M13
REVERSE PR→	Pr#2	VAR2	VAR2	VN°AR1	VAR2	—
H_2O	70.5 29.5	74.4 25.6	77.3 21.7	74.2 25.7	78.7 21.3	78.4 21.6
DNA template	1	0.5	6.5	0.5	0.5	0.5
FORWARD PR	5	5.5	1.6	5.5	1.2	2.6
REVERSE PR	5	1.1	1.1	1.3	1.1	—
10X RXN BUFFER I						
10 mM dNTP	18 µl	18 µl	18	18	18	18
Taq pol +1	0.5	0.5	0.5	0.5	0.5	0.5

Thermocycler Program:
Step cycle: 30 sec @ 95°C
30 sec @ 52°C
3 min @ 72°C } 30 cycles
Delay cycle: 10 min @ 72°C
Soak cycle: 4°C

Master Mix

Rxn buffer I =	7 x 10	70
dATP		14
dCTP		14
dGTP		14
dTTP		14
	TV	126 µl



9 Dec 92 Transformations of 8 Dec 92 didn't produce any colonies. Repeat of transformation w/ INVαF' cells. Ref: MaxBac Baculovirus Expression System Version 1.4.1.
Have One Shot INVαF' competent cells. Each tube contains 50 ul of competent cells.
1. Add 2.5 ul of fresh dilution of 0.5 M βME (530 ul sterile water and 20 ul 14.4 M βME) to each of ~~50 ul~~ 7 tubes. Mix
2. by swirling lightly.
2. Tubes 1-5 are for ligations, tube 6 is the pUC 18 control, tube 7 is a no DNA control. Add 1 ul of ligations to the appropriate tube, 10 pg of pUC 18 (1 ul of a 1:100 dilution in TE) to tube #6. Nothing is added to tube #7.
3. Mix by swirling gently. Incubate tubes on ice for 30 min.
4. Heat shock for 45 sec @ 42°C, then place on ice for 2 min.
5. Add 450 ul of SOC media and shake tubes @ 37°C for 1 H.
6. Plate 100 ul of each transformation on ampicillin plates.
7. Grow plates O/N @ 37°C in an inverted position.

~~10~~9 Dec 92 Replated TOP10F' cells from stab (unused) onto LB plates w/o antibiotics and LB w/ 15 ug/ml tetracycline (Tetracycline - made by George Ludwig 6.25 mg/ml in EtOH. Stored -20°C.

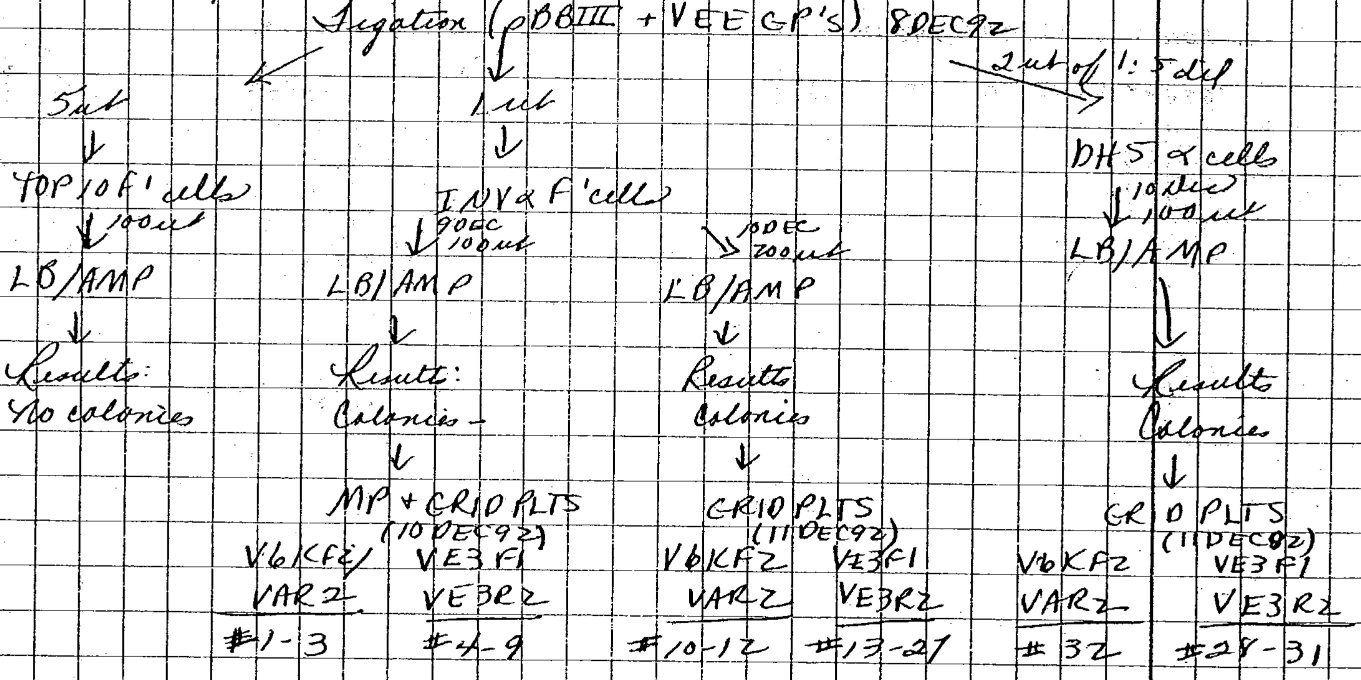
10 DEC 92 Plated 200 ul of transformation w/ INVαF' cells to LB/AMP plates.
No growth on LB/TET plate from TOP10F' cells from stab that was inoculated on 9 Dec 92. Have a 3rd stab tube - plate to LB, LB/TET plates.
Also plated INVαF' cells to LB, LB/TET plates and DH5α to LB, LB/TET plates.

Transformation of DH5α cells: DH5α Competent Cells - Gibco BRL Cat 82636A - Lot# CJO10Z. Amount/vial = 200 ul.
1. Remove competent cells from -70°C freezer, thaw on wet ice.
2. Place 7 - 17x100 mm polypropylene tubes (Falcon 2059) on wet ice.
3. Gently mix cells, then aliquot 100 ul competent cells into chilled polypropylene tubes.
4. To determine transformation efficiency, add 5 ul (0.05 ng) control DNA (pUC19 Monomer (0.01 ug/ml) to one tube containing 100 ul competent cells. Move the pipette through the cells while dispensing. Gently tap tube to mix.
5. For DNA from ligation reactions dilute the reactions

5 fold in 10 mM Tris-HCl (pH 7.5) + 1 mM Na_2EDTA. Add 2 ul of the dilution to the cells (1-10 ng DNA), moving the pipette through the cells while dispensing. Gently tap tubes to mix.

7. Incubate cells on ice for 2 min.
8. Heat shock cells 45 sec in a 42°C water bath. Do not shake.
9. Place on ice for 2 min
10. Add 0.9 ml of R.T. SOC Medium.
11. Shake @ 225 rpm (37°C) for 1 hr.
12. Dilute the reaction containing the plasmid DNA 1:10 with SOC medium. Spread 100 ul of this dilution on LB w/ 100 ug/ml ampicillin and 50 ug/ml X-gal — USED 50 ug/ml AMP No X-gal plates.
13. Incubate ON @ 37°C.

u92 Summary:



TOP10F' cells				INVαF' cells		DH5α	
9DEC92		10DEC92					
LB	LB/TET	LB	LB/TET	LB	LB/TET	LB	LB/TET
+	(−)	+	+	+	(−)	+	(−)

Report Date: 12/11/92

Oligo Primer Design Report

--- SIMPLE PRIMER ANALYSIS V26M-R3

Sequence: 5′-CATTTTGCTGCCGGTCAGTTCTATGTAAGCAGCTTGC-3′

M13/pUC18

Length: 37

Composition: A:6 C:9 G:9 T:13

%GC: 48 %

Tm C: 93 C

Secondary Structure: YES (Energy cutoff: 0.0 kcal)

Runs: YES (Reject runs >= 3 bases)

Em

ym

$= \frac{0.3209 \cdot 101}{391,800}$

$= 0.0000827$

ity = 82.7 pmol/µl

pc

∴ Need 100 pmol/µl = 1.2 µl/rxn.

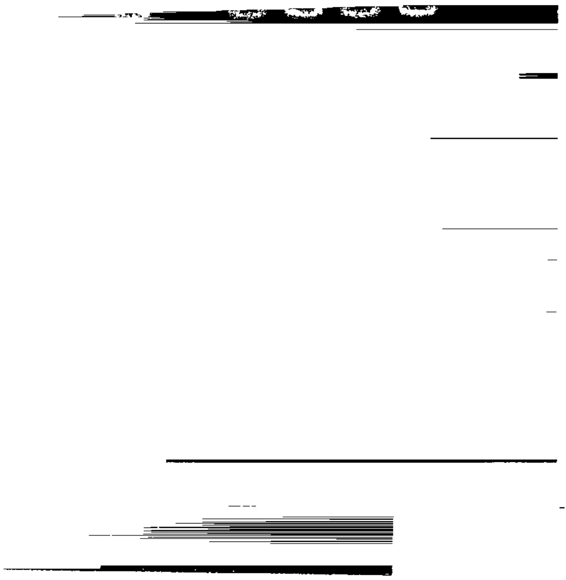
```
     DUAL λ        01          Abs       Factor= 1.000
                                         Read average = 01
 S#     260.0     280.0    λA/λB    S#     260.0     280.0    λA/λB
     5ul in 500ul H2O = 1:101 dil
 1    -0.0000   -0.0001   .2070
 2     0.3209    0.2126   1.509 V26MR3 11DEC92
 3     0.5248    0.2930   1.791 V26MR2 11DEC92

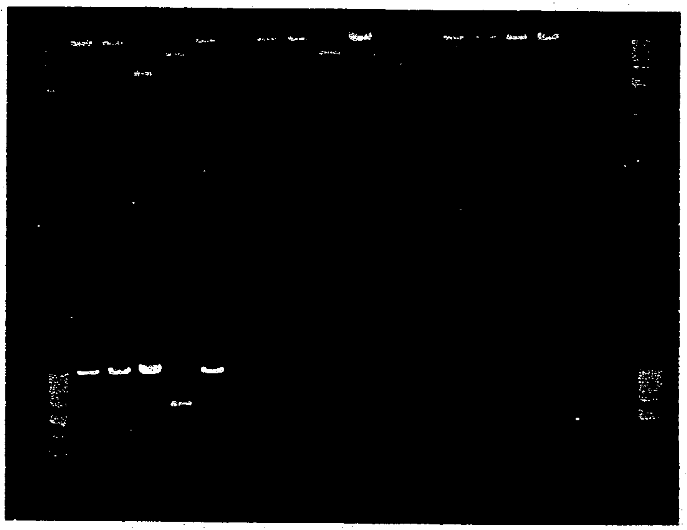
    λ        ABS     SOURCE     12:50    CELL
 260.0     0.0000    UV/Vis   12/11/92    1
```

Minipreps - Promega minicolumn procedure - done by Cathy.

Dec 42 RE analyses

H_2O 14ul
10x React 3 buffer 2ul
Bam HI 1ul
Miniprep DNA 3ul
Inc 37°C for 1H
Add 0.1 Vol of 5M NaCl
Add 1ul Hind III
Inc 37°C for 1H
20ul on 1% LE agarose gel





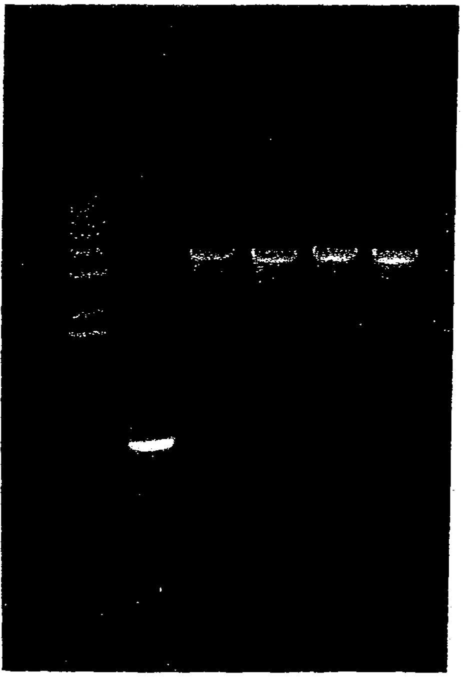
3848
5
3953

1st ROUND PCR
14DEC92

DNA →	λC	20WAT 5	5	12	12
FORWARD PR	Pr #1	V26MF1	V26MF1	V26MF1	V26MF1
REVERSE PR	Pr #2	V26MR2	V26MR3	V26MR2	V26MR3
H_2O (ul)	70.5	78.9	78.5	78.9	78.5
DNA template (ul)	1	0.5	0.5	0.5	0.5
FORWARD PR (ul)	5	1.3	1.3	1.3	1.3
REVERSE PR (ul)	5	0.8	1.2	0.8	1.2
10 x RXN BUFFER (I)	18ul of M.M (I)				
10mM dNTP					
	Vortex, microfuge				
Taq pol (ul)	0.5ul	0.5	0.5	0.5	0.5
TV (ul)	22.5 100	21.1 100	21.5 100	21.1 100	21.5 100
Mineral oil (ul)	100ul				

Thermocycler Program
Step cycle (30 sec @ 95°C
30 sec @ 52°C
35 cycles 3 min @ 72°C
Delay cycle: 10 min @ 72°C
Soak cycle: 4°C

Master Mix (I)
10X Rxn buffer I = 6x10 = 60ul
dATP = 6x 2 = 12 ul
dCTP = " "
dGTP " "
dTTP = "
108



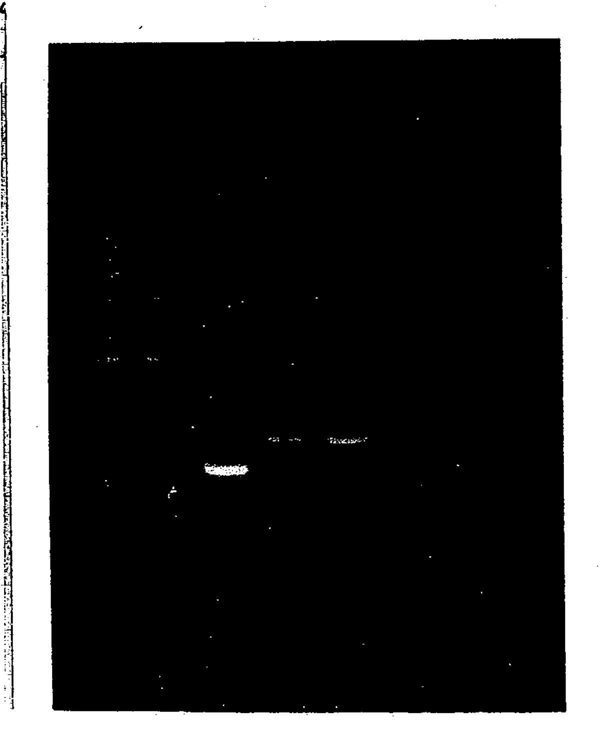
1ST ROUND PCR

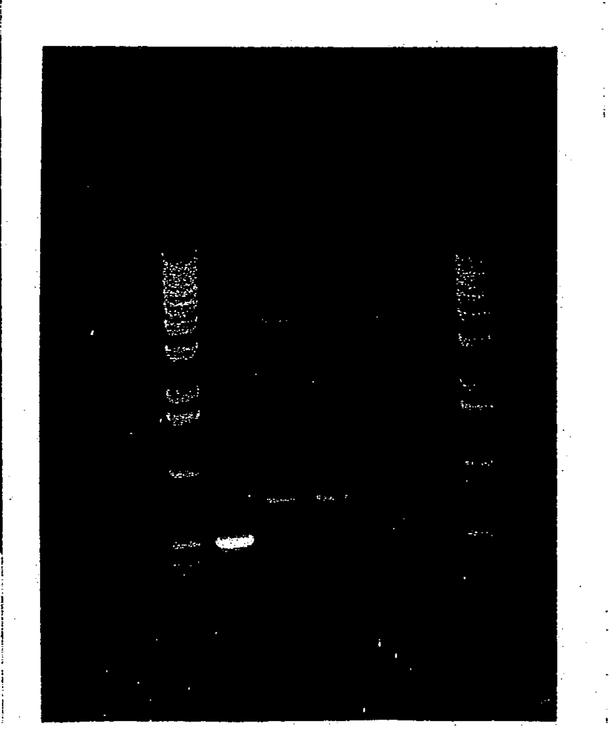
	14DEC92					15DEC92				
DNA	ž C Pr1 Pr2	PCR PRODUCTS Z-5 V26MF1 V26MR2	Z-5 V26MF1 V26MR3	Z-12 V26MF1 V26MR2	Z12 V26MF1 V26MR3	Repeat of 14DEC92				
FORWARD PR	Pr1	M13	M13	M13	M13					
REVERSE PR	Pr2	—	—	—	—					
H_2O (ul)	70.5	78.4	78.4	78.4	78.4	70.5	76.9	76.9	76.9	76.9
DNA template (ul)	1	0.5	0.5	0.5	0.5	1	2.0	2.0	2.0	2.0
FORWARD PR (ul)	5	2.6	2.6	2.6	2.6	5	2.6			
REVERSE PR (ul)	5	—	—	—	—	5	—			
10X RXN BUFFER(I) 10 mM dNTP	18ul					18ul				
	Vortex, microfuge					Vortex				
Taq pol (ul)	0.5	21.6 0.5	0.5	0.5	0.5		0.5 →			
TV										
Mineral oil (ul)	100ul →									

Thermocycler Program:
Step cycle – 35 cycles { 30 sec @ 95°C
30 sec @ 52°C
3 min @ 72°C

Master Mix (I)
10X Rxn Buffer I = 10 × 6 = 60ul
dATP = 2 × 6 = 12ul
dCTP = 2 × 6 = "

Thermocycler program:
Stepcycle 35 cycles { 30 sec @ 95°C
30 sec @ 60°C
3 min @ 72°C
Delaycycle: 10 min @ 72°C





27.5
27.6
20.2
27.2
25.4
[illegible]

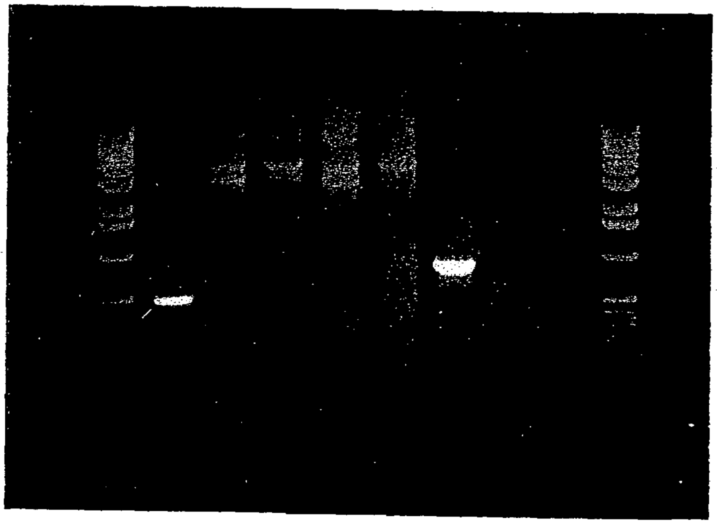
1st ROUND PCR
18DEC92

PCR PRODUCT of 14DEC92

JA	λ 2	20WAT 1/2 V26MF1/V26MR2 (C-100)					
ARI PR	Pr#1	VE3F1	VE3F2	VE3F1	VC F1	VC F1	NC
FR	Pr#2	VAR2	VAR2	VNoARI	VAR2	VCP1	
ED TH(bp)	500	3,095	3,090	2,868	3,914	861	
(ul)	70'	72.5	72.4	73.3	72.?	74.6	
plate	1	5	5	5	5	5	
RD PR	5	1.5	1.6	1.5	1.2	1.2	
FR	5	2.5	2.5	1.3	2.5	0.7	
) (I) TTP	18ul MM(I)						
	Vortex, microfuge						
al.	0.5	0.5	0.5	0.5	0.5	0.5	
TV							
oil							

ocycler program:
ep cycle: 30 sec @ 95°C
30 cycles 30 sec @ 52°C
3 min @ 72°C
lay cycle: 10 min @ 72°C
ak cycle: 4°C

Master Mix (I)
10X Ran buffer I = 7x 10 = 70ul
dATP " = 7x 2 = 14ul
dCTP = " = 14ul
dGTP = " = 14ul
dTTP " = 14ul



B. Ligation - joining of DNA fragments with compatible termini by DNA ligase

  1. T4 DNA ligase - generates phosphodiester linkage between 5' phosphate and 3' hydroxyl; joins cohesive termini or blunt termini

  2. Important parameters (King and Blakesley, 1986)

    a. effective concentration of DNA ends

      1) proximity of ends affects the physical form of the ligation products

      2) typically use 1-10 μg vector/ml reaction (5-20 μl volume)

    b. ratio of vector to insert should be 1:3

      1) higher ratios form concatemers rather than circles

    c. amount of ligase used per reaction (10-100 ng DNA)

      1) sticky end - 0.1 unit

      2) blunt end - 1.0 unit (note: typically use twice the amount of DNA)

    d. reaction temperature

      1) ligation 90% complete at 26°C for 4 h

      2) ligation ~10% complete at 4°C for 4 h, 80% complete at 4°C after 15 h

C. Cloning strategies to reduce background caused by self-ligation of vector

  1. Digestion of vector at a single site followed by dephosphorylation using alkaline phosphatases

    a. DNA ligase requires a 5' phosphate to form a covalent bond

    b. linear DNA molecules transform cells inefficiently

    c. insert DNA will be ligated to vector DNA due to the presence of 5' phosphates on the insert DNA; circular molecules with nicks will be generated

  2. Dephosphorylation of DNA (options)

    a. Calf Intestinal Alkaline Phosphatase (CAIP) ~~65~~°C 37°C

b. Bacterial Alkaline Phosphatase (BAP) 65°C

c. Arctic Shrimp Alkaline Phosphatase (ASAP) 4°C

3. Double digestion of vector DNA with two restriction endonucleases that generate noncompatible termini

   a. ligation of insert DNA generated by the same two enzymes results in forced orientation of insert

   b. double digestion within the pUC 19 multiple cloning site (Table 4)

   c. second digestion efficiency can be monitored by kinasing with [γ-^{32}P]ATP after the first digestion and analyzing second digestion by fractionating on an agarose gel (see M13 Cloning/Dideoxy Sequencing Manual)

   d. can be monitored by self-ligation efficiency

D. Strategies for joining DNA fragments with noncompatible termini

1. Production of blunt ends for blunt end ligation

   a. 3' recessed end - use large fragment of E. coli DNA polymerase I (Klenow fragment) to "fill-in" recessed end

$$\begin{array}{l} 5'\text{——}3' \\ 3'\text{————}5' \end{array} \xrightarrow[\text{dNTPs}]{\text{Klenow fragment}} \begin{array}{l} 5'\text{————}3' \\ 3'\text{————}5' \end{array}$$

55°C. No ethidium bromide has been added to this solution.

2. Prepare the apparatus as demonstrated by the instructor.
3. Cast the gel on a level surface.
4. Pipette agarose onto the gel tray (~20-25 ml).
5. Insert the 8 well 0.8 mm comb. Each well can hold 12-15 µl.
6. Allow the gel to solidify (>15 min).
7. Remove gel casting dams and add enough buffer (100 to 150 ml of 1X TBE) to fill both chambers and flood the surface of the gel (~3 mm depth over gel).
8. Leave the combs in the gel until samples are ready for loading.
9. Carefully remove the comb with a gentle rocking motion.

Solutions: 1X TBE (Tris-borate/EDTA): 100 mM Tris-HCl, 90 mM Boric acid, 2 mM Na_2EDTA (pH 8.3)

C. VECTOR DEPHOSPHORYLATION (Monday AM)

1. Heat S2 at 65°C for 10 min to inactivate the restriction enzyme.
2. Add ~~60~~ 80 µl dH_2O to S2, vortex. use only [illegible]0 µl
3. Label 2 microfuge tubes S3 and S4.
   - Transfer a 5 µl aliquot from S2 to S3.
   - Transfer a 5 µl aliquot from S2 to S4.
   - Save S3 and S4 at -20°C overnight (a rack will be provided). These plasmid controls will be used later. DO NOT LOSE THESE SAMPLES.
4. To S2
   - Add 2 µl of BAP (Bacterial Alkaline Phosphatase) (~280 units).
   - Incubate 65°C for 60 min (return to lecture).

TIME IN: 12:50 TIME OUT:______

5. After lecture: Remove **tubes** S1 **and** S2 from water baths and place on ice.

D. PURIFICATION OF DNA WITH GLASSMAX DNA ISOLATION SPIN CARTRIDGE SYSTEM (Monday PM) This procedure will be demonstrated by your instructor.

1. Preheat 200 µl of TE buffer to 65°C in a water bath (to be used later).

2. Add binding solution (4.5 volumes) to S1 and S2

   S1 - 135 µl
   S2 - 450 µl

3. Assemble and label the microfuge tubes and cartridges appropriately.

4. Transfer S1 and S2 samples to the spin cartridge unit.

   - Centrifuge 13,000 x g for 20 seconds.

5. With gloved fingers, remove the spin cartridge and set on a fresh Kimwipe.

   - Discard the flow through. Replace the cartridge into the same tube.

6. Add 400 µl cold wash buffer to the spin cartridge.

   - Centrifuge 13,000 x g for 20 s.

7. Remove the cartridge, discard the wash solution and replace the cartridge.

   - **Repeat** this wash step **two** more times.

8. After removing the final wash buffer, centrifuge the cartridge for 1 min. to remove all traces of buffer.

9. Label a new recovery tube. (An ordinary microfuge tube is not compatible with the column - the cover won't fit.)

10. Transfer the spin cartridge insert into the new tube.

11. Add preheated TE buffer to the spin cartridge.

    S1 - 40 µl TE
    S2 - 100 µl TE

12. Centrifuge 13,000 x g for 20 s to elute DNA.

13. Discard cartridge. Store DNA on ice.

Solutions

Binding solution - 6M NaI
TE - 10 mM Tris-Cl (pH 8), 1 mM EDTA

F QUANTITATION OF DNA BY AGAROSE GEL ELECTROPHORESIS (Monday PM)

1. Label two new microcentrifuge tubes $S1^{*}$ and $S2^{*}$ and add the following:

   20 μl sterile dH_2O
   2 μl Endo R Stop buffer

2. Add 2 μl of the digested DNA from step 1 to the matching tube.

$$S1 \longrightarrow S1^{*}$$
$$S2 \longrightarrow S2^{*}$$

3. The instructor will provide a tube of *Hin*d III digested λ DNA for use as a size and concentration standard.

   - **Incubate λ DNA at 65°C for 5 min, then immediately cool on ice.**

   (see the product information sheet at the end of this section)

4. You will also be provided with tubes of **uncut** pSGlobin and pUC19.

5. Load the 0.8% agarose gel with samples and volumes indicated below.

Lane	Volume	Sample
1	12 μl	λ *Hind* III DNA
2	12 μl	*Pst* I/*Ava* I pSGlobin (from $S1^{*}$)
3	12 μl	*Pst* I/*Ava* I pSGlobin (from $S1^{*}$)
4	12 μl	Pst I pUC19 (from $S2^{*}$)
5	12 μl	Pst I pUC19 (from $S2^{*}$)
6	12 μl	λ *Hind* III DNA
7	12 μl	pSGlobin **uncut** (50 ng)
8	12 μl	pUC19 **uncut** (50 ng)

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